AD-A142 144

20000803102

(12)

AD

TOXICITY OF TNT WASTEWATERS TO AQUATIC ORGANISMS

Final Report

Volume I

Acute Toxicity of LAP Wastewater and 2,4,6-Trinitrotoluene

By

DAVID H. W. LIU
RONALD J. SPANGGORD
HOWARD C. BAILEY
HAROLD S. JAVITZ
DAVID C. L. JONES

MARCH 1983
~~June 1984~~

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21701

Contract DAMD17-75-C-5056
SRI International
333 Ravenswood Avenue
Menlo Park, CA 94025

Project Officer: J. Gareth Pearson
U.S. Army Medical Bioengineering Research
and Development Laboratory
Fort Detrick, Frederick, MD 21701

DTIC
ELECTE
JUN 15 1984
S D
B

DTIC FILE COPY

DoD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Reproduced From
Best Available Copy

84 06 13 027

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A142 144 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) TOXICITY OF TNT WASTEWATERS TO AQUATIC ORGANISMS Volume I - Acute Toxicity of LAP Wastewater and 2,4,6-Trinitrotoluene	5. TYPE OF REPORT & PERIOD COVERED Final Report, 15 April 1975 to 15 December 1978
	6. PERFORMING ORG. REPORT NUMBER LSU-4262
7. AUTHOR(s) David H. W. Liu, Ronald J. Spanggord, Howard C. Bailey, Harold S. Javitz, and David C. L. Jones	8. CONTRACT OR GRANT NUMBER(s) DAMD 17-75-C-5056
9. PERFORMING ORGANIZATION NAME AND ADDRESS SRI International 333 Ravenswood Avenue Menlo Park, CA 94025	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62720A.3E162720A835.AA.045.
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE March 1983
	13. NUMBER OF PAGES 85
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) US Army Medical Bioengineering Research and Development Laboratory Fort Detrick, Frederick, MD 21701	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE N/A

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Acute toxicity	Photolysis	LAP wastewater
Incipient LC50	Water quality	2,4,6-Trinitrotoluene
Fish	Toxic interactions	RDX
Invertebrates	Bioconcentration	1,3,5-Trinitrobenzene
Algae	Fish eggs and larvae	2,4,6-Trinitrobenzaldehyde

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The acute toxicity to aquatic organisms of Composition B (COMP B) type LAP wastewater was determined. The tests were performed primarily on a 1.6-to-1 mixture of TNT and RDX, which are the major organic components of the wastewater and which are normally present in a 1.6:1 ratio in untreated wastewater. Acute toxicity tests were also performed on TNT and RDX, and on 2,4,6-trinitrobenzaldehyde, 2,4,6-trinitrobenzonitrile, 1,3,5-trinitrobenzene, and 4,6-dinitroanthranil, which are phototransformation products of TNT and RDX.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. KEY WORDS (Continued)

2,4,6-trinitrobenzonitrile
4,6-dinitroanthranil

20 ABSTRACT (Continued)

All of these tests were conducted to obtain an initial assessment of the potential hazard of LAP wastewater to aquatic life.

Incipient LC50s--obtained in flow-through tests with four species of fish and two species of invertebrates and based on measured concentrations--ranged from 0.19 to 13.9 mg/L for TNT and 0.17 to 16.2 mg/L for the TNT-RDX mixture. Bottle tests performed with four species of algae indicated that the algae were less sensitive to TNT and the mixture than were the most sensitive animal species.

Exposure of LAP wastewater, TNT, and the TNT-RDX mixture to filtered UV light (simulated sunlight) reduced their toxicity by a factor of up to 25. The toxicity of these materials decreased as the photolytic degradation of TNT increased. The phototransformation products of TNT were generally more toxic than TNT; however, there is evidence that when TNT photolyzes, the concentrations of the photoproducts do not reach lethal levels.

Water temperature, pH, and hardness slightly modified the toxicity of TNT and the TNT-RDX mixture. Some of these parameters increased the toxicity of these materials and some reduced their toxicity; however, none of these parameters caused the toxicity of the test materials to change by more than a factor of two.

Tests on various mixtures of TNT and RDX provided evidence that they interact toxicologically; however, the magnitude of interaction was low. Antagonism was exhibited in all mixtures (including the 1.6:1 mixture) except the 3:1 mixture, which showed slight synergism.

Data from the exploratory bioconcentration tests were inconclusive. The bioconcentration factor (BCF) estimated from computed log P value for TNT indicated that it has little propensity to bioconcentrate.

Tests on the aqueous and benzene fractions of LAP wastewater and a partially photolyzed solution of TNT indicated that the most toxic components of both materials are nonpolar.

FOREWORD

The U.S. Army Medical Research Development Command, Ft. Detrick, Frederick, MD, is conducting a research program for the purpose of developing the scientific data base necessary for assessing the potential environmental hazards associated with compounds unique to the munitions industry. From these data, criteria will be developed that are qualitative or quantitative estimates of the concentrations of a pollutant in ambient waters that, if not exceeded, should ensure the protection of aquatic organisms and human health. These criteria, when compared to actual or estimated environmental concentrations, will form the basis of a hazard assessment. In addition, these criteria will be used to assess the adequacy of current pollution abatement technologies and thus influence research and development in this area.

This report represents a portion of the data base being developed on TNT and its associated wastewaters and should not be construed as a complete evaluation or as official policy of the U.S. Army Surgeon General.

This work was conducted under the technical control and review of the U.S. Army Medical Bioengineering Research and Development Laboratory: J. Gareth Pearson and William H. van der Schalie (Aquatic Toxicology), Jesse J. Barkley, Jr. (Analytical Chemistry), and Jerry W. Highfill (Statistical Analysis).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Accession For	
NTIS GRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
PER CALL TC	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

DTIC COPY INSPECTED 3

EXECUTIVE SUMMARY

This report is the first in a series of four reports on the toxicity of TNT wastewaters to aquatic organisms. The information presented in the four reports was developed in a study performed by SRI International for the U.S. Army Medical Research and Development Command under Contract No. DAMD17-75-C-5056. The study was undertaken to assist USAMRDC in developing a data base for assessing the potential hazards to to aquatic life of wastewater from trinitrotoluene (TNT) manufacturing and processing plants.

This report presents and discusses the results of acute toxicity tests and exploratory bioconcentration tests performed on wastewater produced by load, assemble, and pack (LAP) facilities that handle an explosive mixture known as Composition B (COMP B). It also presents and discusses acute toxicity data obtained from tests on TNT and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), which are the major, militarily unique organic constituents of LAP wastewater, and on four phototransformation products of TNT.

Conclusions about the acute toxicity of LAP wastewater are based primarily on toxicity tests performed on synthetic LAP wastewater (called LAP water), which, after extensive chemical analysis and characterization of authentic wastewater samples, was defined as a 1.6-to-1 mixture of TNT and RDX.

The results of flow-through acute toxicity tests with the invertebrate Daphnia magna indicate that LAP wastewater and TNT can be highly toxic to some aquatic organisms. With D. magna, we obtained incipient LC50s (defined as the concentration of a substance above which 50 percent of the test organisms cannot survive indefinitely) of 0.17 and 0.19 mg/L for the wastewater and TNT, respectively. The incipient LC50s obtained with four species of fish (fathead minnow, bluegill sunfish, channel catfish, and rainbow trout) ranged from 1.4 to 1.8 mg/L for TNT and from 1.5 to 3.3 mg/L for LAP water. These incipient LC50s were based on measured test concentrations. With algae (four species were tested), the lowest nominal concentration that caused a statistically significant ($p \leq 0.05$) effect on population growth was 4.1 mg/L for TNT and 0.6 mg/L for LAP water. During the algal assays, noticeable photolysis of TNT occurred; thus, we consider the toxicity data questionable. LAP water was more toxic to fathead minnows during the first week after hatching than to the unhatched embryo and to older fish. With 2- and 7-day-old minnows, the 96-hour LC50s of LAP wastewater were 1.1 and 0.7 mg/L (nominal), respectively.

The acute toxicity of LAP wastewater and TNT can be reduced substantially by exposing these materials to simulated sunlight, however,

the reduction in toxicity is not appreciable unless relatively extensive photolysis of TNT occurs. Tests performed with four species each of fish, invertebrates, and algae showed that about 99 percent reduction in concentration of TNT by photolysis can result in decreases up to 25-fold in the acute toxicity of LAP wastewater and TNT. Although it has been reported that pH can affect the rate of photolysis of TNT and the kinds of phototransformation products produced, we found no appreciable difference between the toxicity of LAP wastewater or TNT photolyzed at pH 5, 7, and 9.4.

Acute toxicity tests performed on the aqueous and benzene fractions of LAP wastewater and solutions of partially (about 50 percent) photolyzed TNT indicated that the most toxic constituents in the wastewater and solutions of photolyzed TNT are nonpolar (i.e., they were present in the benzene fraction). TNT and RDX are nonpolar compounds and known constituents of LAP wastewater. Nonpolar compounds that might have been present in the solution of partially photolyzed TNT include TNT and some of its phototransformation products, such as 1,3,5-trinitrobenzene (TNB), 2,4,6-trinitrobenzaldehyde (TNBA), and 4,6-dinitroanthranil (DNAN). 2,4,6-trinitrobenzonitrile (TNBN) may not have been present because TNT was irradiated at pH 7 and that compound is reportedly not produced unless irradiation occurs at a pH of less than 5.

Although we obtained conclusive evidence that exposure to simulated sunlight reduces the toxicity of LAP wastewater and TNT, we found TNB, TNBA, TNBN, and DNAN to be more toxic than TNT to fathead minnows and D. magna. DNAN was the most toxic compound; its 96-hour LC50 (minnows) and 48-hour EC50 (daphnids) were 0.16 and 0.34 mg/L, respectively. We obtained some evidence that the reason TNT and LAP wastewater exhibited less toxicity after photoirradiation was that none of these phototransformation products accumulated to lethal levels while TNT or the wastewater were being irradiated.

Water temperature, pH, and hardness are known to affect the toxicity of some chemicals. These factors had a statistically significant effect on the toxicity of photolyzed and nonphotolyzed LAP water; however, none of them cause more than a 2-fold change in the acute toxicity of these materials. We considered their effects to be environmentally insignificant.

Toxicity tests on 1:1, 1:3, 3:1, and 1.6:1 mixtures of TNT and RDX indicated that these compounds are slightly antagonistic when present in ratios of 1:1, 1:3, and 1.6:1 and slightly synergistic when mixed in a ratio of 3:1. The degrees of synergism and antagonism were very low, and in our opinion biologically insignificant.

Exploratory bioconcentration tests (4-day exposures) performed with two invertebrates and one species of algae and fish on TNT, RDX, and a 1.6:1 mixture of TNT and RDX indicated that neither compound, alone or together, bioconcentrates extensively enough to cause concern. TNT bioconcentrated more than RDX. The 4-day bioconcentration factors (BCF)

for TNT ranged from 9.5 for fish muscle to 453 for the algae; for RDX, the 4-day BCFs ranged from 1.6 for D. magna to 123 for the algae.

The data presented in this report were not adequate for recommending water quality criteria for LAP wastewater or TNT. The high acute toxicity of these substances suggests that both should be evaluated for subchronic and/or chronic toxicity. Data from early life stage tests (subchronic) with fathead minnows and rainbow trout and life-cycle tests (chronic) with D. magna and fathead minnows are presented and discussed in Volume III.

CONTENTS

CONTENTS (Concluded)

TABLES

TABLES (continued)

TABLES (concluded)

ILLUSTRATION

INTRODUCTION

The production of munitions compounds generates a significant volume of wastewater, which has historically been discharged into the environment with little or no treatment. In order to assess the hazard of these wastewaters to human health and to aquatic life, the U.S. Army Medical Research and Development Command (USAMRDC) funded a comprehensive investigation to develop a scientific data base composed of data from literature reviews, on-site field studies, and laboratory investigations in mammalian and aquatic toxicology.

Of the various kinds of wastewaters produced in the manufacture and processing of munitions compounds, condensate and LAP wastewater are of major concern to the USAMRDC. Condensate wastewater is produced during the manufacture of 2,4,6-trinitrotoluene (TNT) and comprises at least 30 organic compounds, with 2,4-dinitrotoluene (DNT) accounting for almost 50 percent of the total dissolved organics. LAP wastewater is produced at load, assemble, and pack (LAP) facilities during the washing of shells and other equipment. The composition of LAP wastewater depends on the particular kind of explosive formulation being processed by the LAP facility. The LAP wastewater of primary concern is that produced by LAP facilities handling an explosive formulation called COMP B. That wastewater is composed primarily of TNT and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX).

Under Contract DAMD 17-75-C-5056, SRI International conducted a laboratory study to determine the acute, subchronic, and chronic toxicity to aquatic organisms of condensate wastewater, LAP wastewater from COMP B processing plants, and selected organic components of both wastewaters. The study comprised four phases, each with several tasks, and followed the approach proposed by Pearson and coworkers (1979) for toxicological evaluation of complex industrial wastewaters.

The results of SRI's study are presented in a series of four reports. This report is the first in the series; it presents and discusses the acute toxicity data on LAP wastewater and TNT and the results of an exploratory bioconcentration study on TNT and RDX. It also contains two general sections—one that briefly describes the four phases and the various tasks and another that describes the facilities, equipment, and procedures used to conduct the acute toxicity and exploratory bioconcentration tests. The Appendix to Volume I contains computer printouts that present for each test with animals the tested concentrations, the number of animals in each treatment group, the number of animals that responded, and the LC50s and their 95 percent confidence limits. The appendix also contains graphs of the response of the algae to the tested substances.

The other reports in the series are: Volume II, "Acute Toxicity of Condensate Wastewater and 2,4-Dinitrotoluene"; Volume III, "Subchronic and Chronic Toxicity of LAP Wastewater and 2,4,6-Trinitrotoluene"; and Volume IV, "Subchronic and Chronic Toxicity of Condensate Wastewater and 2,4-Dinitrotoluene."

BACKGROUND

Source and Chemical Characteristics of LAP Wastewater

LAP wastewater is generated at facilities that load, assemble, and pack (LAP) TNT-based explosives into munitions; it results from rejected shell and equipment washing and house-cleaning activities. Such facilities are present at several Army Ammunition Plants (AAPs), including Milan, Louisiana, Iowa, Lone Star, Joliet, and Holston AAPs. The chemical composition of LAP wastewater varies with the specific explosive formulation handled at an LAP facility. The LAP wastewater evaluated in this study is the type produced at facilities that handle COMP B (probably the most common of the TNT-based explosives), which is a mixture of 60 percent TNT and 40 percent waxed RDX.

At some AAPs, LAP wastewater is treated before it is discharged. Treatment consists of passing the effluent through diatomaceous earth to remove undissolved materials and activated carbon columns to remove organic substances. Analysis of numerous samples of untreated COMP B LAP wastewater showed that it contains primarily TNT and RDX in the ratio of 1.6 parts TNT to 1.0 part RDX (Spanggord et al., 1978). However, after the wastewater enters the aquatic environment, the ratio of these two components can become highly dynamic, principally because of the high photoreactivity of TNT.

Ruchhoft and coworkers (1945) and Nay and associates (1972) found that TNT is photolyzed more rapidly under alkaline than under acid conditions. The photolysis of TNT creates what is commonly called "pink water." Burlinson and coworkers (1973) identified 2,4,6-trinitrobenzaldehyde, 2,4,6-trinitrobenzonitrile, 1,3,5-trinitrobenzene, and 4,6-dinitroanthranil as the major photoproducts of TNT. However, 2,4,6-trinitrobenzonitrile was formed only when the TNT solution was not buffered and the pH drifted below 5 during photoirradiation.

Toxicity of LAP Wastewater, TNT, and RDX

Before this study was initiated, the toxicity of LAP wastewater *per se* was unknown. Although considerable data had been developed on the effects of RDX on aquatic life, not much was known about the acute toxicity of TNT to aquatic organisms.

Pedersen (1970) determined the acute toxicity of TNT to bluegills in moderately soft (90 mg/L $CaCO_3$) and moderately hard (180 mg/L $CaCO_3$) water at temperatures of 10 and 25°C. He found that water hardness and temperature had little effect on the toxicity of TNT. The 96-hour LC50s ranged from 2.3 to 2.8 mg/L. As a result of this work, the author concluded that a water quality standard of 5 mg/L TNT was too high and an interim standard of 0.26 mg/L should be adopted pending the results of more definitive

bioassays. Gring (1971) reported 96-hour LC50s for TNT of 2.0 to 2.6 mg/L in bluegills. LeClerc (1960) performed a study similar to Pedersen's, but his primary interest was in the effect of water hardness on TNT toxicity. At a temperature of 21 to 23°C and with water hardnesses of 12.5 and 160 mg/L $CaCO_3$, the 96-hour LC50s were about 4 to 5 mg/L; hence, as in Pedersen's study, water hardness had no effect on the toxicity of the compound.

Matthews et al. (1954) found that nonphotolyzed TNT was not toxic to goldfish at concentrations of 2 mg/L and higher. At 2 mg/L, the photolyzed solution (which they called colored TNT complex) caused 25 percent mortality (one of four fish died). The validity of these results is questionable because of the small test population.

Schott and Worthley (1974) determined the toxicity of 2,4,6-trinitrotoluene to duckweed (Lemna perpusilla) at two levels of pH. The exposure period was 11 days. At pH 6.3, no effects occurred at 0.5 mg/L; however, the plant was affected at 1.0 mg/L. At pH 8.5, the plant was not affected at 0.1 mg/L; but, it was affected at 1.0 mg/L.

Smock and coworkers (1976) also studied the toxicological properties of TNT before and after its photoirradiation. The photolyzed solution was prepared by adjusting its pH to 9.7 and exposing it to fluorescent light at an intensity of 4300 lumens/m^2 at 24°C for one week. Growth of the algae Selenastrum capricornutum and Microcystis aeruginosa was inhibited by nonphotolyzed TNT at 5 and 15 mg/L, respectively. Photolyzed TNT was less toxic. It inhibited the growth of S. capricornutum at 9 mg/L; and although it was lethal to M. aeruginosa at 50 mg/L, it stimulated growth at lower concentrations. Smock et al. (1976) also reported 96-hour LC50s in the fathead minnow (tested under flow-through conditions) of 2.6 mg/L for nonphotolyzed TNT and 1.6 mg/L for photolyzed TNT. No effects were observed on minnows exposed to 0.05 mg/L TNT and 0.07 mg/L photolyzed TNT.

Won and coworkers (1976) evaluated TNT and several of its degradation products for toxicity to an alga (Selenastrum capricornutum), a marine copepod (Trigriopus californicus), and the larvae of an oyster (Crastrea gigas). They also determined the toxicity and mutagenicity of the chemicals to the bacterium Salmonella typhimurium. At 2.5 mg/L, TNT suppressed growth in the alga. That concentration and 10.0 mg/L increased mortality of the copepod and oyster larvae. TNT was mutagenic at a concentration of 0.5 mg/L. At 5.0 mg/L, 2,6-dinitro-4-hydroxyaminotoluene, 2,4-dinitro-6-hydroxyaminotoluene, 2,2',4,4'-tetranitro-6,6'-azoxytoluene, and 2,2',6,6'-tetranitro-4,4'-azoxytoluene were neither toxic nor mutagenic in S. typhimurium. In the same organism, no toxic or mutagenic response was observed at 50 mg/L of 2,6-dinitro-4-aminotoluene, 2-nitro-4,6-diaminotoluene, and 2,4-dinitro-6-aminotoluene.

Bentley et al. (1978) performed a comprehensive study on the toxicity of RDX to aquatic organisms, using essentially the same approach we used on TNT wastewater. They found RDX to be nontoxic to four species of algae

at concentrations as high as 32 mg/L (nominal). In tests with four species of invertebrates, the estimated 48-hour EC50 was greater than 100 mg/L (nominal). In tests with four species of fish, the estimated 96-hour LC50s ranged from 4.1 to 6.0 mg/L. RDX did not accumulate significantly in the tissues of fish exposed to the compound for 28 days. Chronic toxicity tests with the fathead minnow showed no statistically significant effects at 3.0 mg/L. The authors recommended a water quality criterion of 0.35 mg/L for RDX.

Sullivan and coworkers (1979) concluded that for RDX, a 24-hour average concentration of 0.30 mg/L would not be harmful to aquatic life. This conclusion was based on a review of the literature on the acute and chronic effects of RDX to fish and invertebrates and on algal toxicity studies.

Field Studies

USAMRDC has sponsored several on-site field studies of the TNT wastewater problem. One study surveyed the biota in water bodies receiving the effluent from Joliet Army Ammunition Plant (JAAP). It was found that the water content of TNT above and below the LAP wastewater outfall in Prairie Creek was less than 0.5 μg/L, whereas in Doyle Lake, which also receives LAP wastewater, up to 27 μg/L TNT was found. [LAP wastewater from JAAP is treated with activated carbon before release which may account for the relatively low level of TNT found in the receiving streams.] The study found little or no difference in the abundance and diversity of the macroinvertebrate and algal population above and below the Prairie Creek outfall; the biota in Doyle Lake were not studied (Dr. W.H. van der Schalie, USAMRDC, personal communication).

Stillwell and coworkers (1976) studied the biota in Doyle Lake at JAAP and in Prairie Creek. In the spring, they found up to 66 μg/L TNT in the stream entering the lake; that stream receives the LAP effluent. In the fall, the stream contained up to 30 μg/L TNT. In both seasons, water in the lake contained less than 1 μg/L TNT, but the sediment contained up to 43 μg/L. They found evidence of stress in the macroinvertebrate and algal populations in both the stream and lake, with the stress being most evident in the stream and in the portions of the lake closest to the outfall. In Prairie Creek, they found up to 35 and 40 μg/L TNT in the water and sediment, respectively. The creek was studied only during the fall when the water flow was low. They observed some differences in the invertebrate populations above and below the outfall, but they were not sure of the cause. The algal bloom observed downstream of the outfall was believed to be caused by the effluent.

Weitzel and associates (1975) studied the distribution and abundance of biota in three creeks that receive LAP effluent from the Iowa Army Ammunition Plant (IAAP). In water samples from Long, Spring, and Brush Creeks, they found TNT levels of up to 3, 3.4, and 1.5 μg/L, respectively; sediment samples contained up to 1, 0.1, and 617 mg/kg, respectively. Small amounts of other munitions-related compounds were also found in the water and sediment. Like JAAP, IAAP treats its effluent with activated

carbon (Dr. W.H. van der Schalie, USAMRDC, personal communication), which probably explains the low levels of TNT and other nitroaromatics in the water in the creeks. Although these investigators found variations in the distribution and abundance of biota in the creeks, they could not discern from the data whether the variations were caused by the presence of the munitions compounds or by other factors.

At the Milan Army Ammunition Plant (MAAP), which is primarily a LAP facility and does not treat its waste effluents, Huff and coworkers (1975) reported finding less than 0.1 mg/L TNT in water downstream of the effluent outfalls on the Rutherford Fork of the Obion River. The water also contained less than 0.2 mg/L 1,3,5-trinitrobenzene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene. These compounds were the only munitions-related compounds that Huff and coworkers quantified, but they used relatively insensitive analytical techniques. They concluded that the wastewater from MAAP had no apparent effect on the biota in the stream. However, they stated that their ability to detect effects was hampered by stream conditions and other factors.

GENERAL EXPERIMENTAL APPROACH

The experimental approach presented in this section applies to the whole study. Data presented in this report were obtained after performing all of the tasks in Phases I and II and Task I of Phase III.

To evaluate condensate and LAP wastewater for toxicity to aquatic organisms, we used the approach described by Pearson and coworkers (1979) for use with complex industrial wastewater. The approach differs from the traditional one of basing the hazard assessment primarily on the evaluation of toxiological information obtained on individual components in the wastewater in that major emphasis is placed on the results of toxicity on tests a synthetic but representative wastewater mixture, single compounds are evaluated for toxicity only when necessary.

The aquatic toxicological testing scheme associated with the approach comprises three phases, each with four tasks. We added a three-tasked phase to the beginning of the scheme to evaluate authentic condensate and LAP wastewater for toxicity and added one task to each of the original phases. A brief description of each phase and task follows.

Phase I - Preliminary Studies on Authentic Wastewaters and Selected Components Thereof

The objectives of Phase I were to determine the extent to which photolysis affects the toxicity of authentic LAP and condensate wastewaters, TNT, and DNT; to determine whether the toxicity of the wastewaters and photolyzed TNT and DNT is caused primarily by the polar or nonpolar compounds; to determine whether toxicological interactions occur between TNT and RDX—the major components of LAP wastewater; and to determine the acute toxicity of selected organic constituents of the two wastewaters. The acute toxicity tests in this phase were not replicated and were conducted under static conditions.

Task 1—Task 1 entailed determining the effect of filtered ultraviolet (UV) irradiation (stimulated sunlight) on the acute toxicity of TNT, DNT, and condensate and LAP wastewaters to the fathead minnow. It included determining whether pH during UV irradiation affects acute toxicity.

Task 2—The purpose of Task 2 was to determine the relative toxicity to the fathead minnow and *Daphnia magna* of the aqueous and benzene fractions of the wastewaters and 50 percent photolyzed TNT and DNT, and thus identify whether the most toxic components of these materials are polar or nonpolar compounds.

Task 3--The purpose of Task 3 was to determine the acute toxicity to the fathead minnow and D. magna of selected components identified in LAP and condensate wastewaters. The LAP components selected were TNT, RDX, and four phototransformation products of TNT. Thirty-three condensate wastewater components were tested, with the objective of determining which of them should be included in the synthetic blend to be used in subsequent phases of the study. Task 3 also included determining whether TNT and RDX interact toxicologically.

Phase II - Studies on Synthetic Wastewaters and Additional Studies on Selected Components

The objective of Phase II was to further delineate the toxicological properties of TNT, DNT, and LAP and condensate wastewaters. In this phase, the authentic wastewaters were replaced with synthetic blends, which were called LAP and condensate water instead of wastewater. All tests were conducted under static conditions. The tests with the animal species were conducted in duplicate. The tests with algae were performed primarily in triplicate, with some concentrations in some of the tests being run in quadruplicate. The blends were formulated after chemical analysis of a large number of LAP and condensate wastewater samples and toxicological screening tests, including bacterial mutagenesis studies (Ames test). The samples were analyzed and the Ames tests were performed by SRI under Contract No. DAMD 17-76-C-6050, and the respective results were reported by Spanggord and coworkers (1978) and Dilley and coworkers (1979).

Task 1--The purpose of Task 1 was to standardize the irradiation procedures for preparing photolyzed LAP and condensate waters. The data used to establish the standard for each blend were obtained by conducting acute toxicity tests with D. magna on aliquots of the blends after each was exposed to simulated sunlight for different times and analyzed for the original components.

Task 2--The purpose of Task 2 was to determine the acute toxicity of photolyzed and nonphotolyzed LAP and condensate waters, TNT, and DNT to four species of fish, four species of aquatic invertebrates, and four species of algae to identify the most sensitive species in each trophic level.

Task 3--Task 3 entailed determining the influence of water temperature, hardness, and pH on the acute toxicity of the materials used in Task 2 to the most sensitive warm-water fish.

Task 4--The purpose of Task 4 was to determine the acute toxicity of photolyzed and nonphotolyzed LAP and condensate waters to selected early life stages of the fathead minnow under the water quality conditions associated with the highest toxicity found in Task 3.

Task 5--The purpose of Task 5 was an exploratory bioconcentration study of TNT, RDX, and DNT. The study was performed with bluegill sunfish, D. magna, Lumbriculus variegatus, and S. capricornutum, using 4-day exposures.

Phase III - Definitive Acute Toxicity Studies

The tasks in Phase III were essentially the same as those in Phase II, but the tests were performed under flow-through conditions and the test materials were LAP and condensate waters, TNT, and DNT.

Task 1--In Task 1, time-independent acute toxicity tests were conducted using four species of fish and two species of invertebrates to determine the incipient LC50s of the test materials.

Task 2--The purpose of Task 2 was to conduct tests under flow-through conditions and thus confirm the effect of water quality on the acute toxicity of the test materials to the most sensitive fish species. This task was to be performed only if relatively large and consistent effects were observed under static test conditions (Phase II, Task 3).

Task 3--The purpose of Task 3 was to determine the effect of continuous exposure to the test materials on egg hatchability and on fry growth and survival in channel catfish and rainbow trout.

Task 4--Task 4 was reserved for additional studies on the toxicity of the photolyzed synthetic wastewater blends.

Task 5--The purpose of Task 5 was to conduct full-scale bioconcentration tests on TNT and DNT.

Phase IV - Chronic Toxicity Studies

Phase IV comprised five tasks, three of which were long-term exposure studies with fish and invertebrates. These tasks represented the final scheduled step in the toxicological evaluation of TNT wastewater. The test materials were photolyzed and nonphotolyzed LAP and condensate waters, TNT, and DNT.

Task 1--The purpose of Task 1 was to determine the effects of the test materials on the survival and reproduction of *D. magna*, using one-generation chronic exposure tests.

Task 2--The purpose of Task 2 was to conduct 30-day, early life stage toxicity tests with fathead minnows to determine the range of concentrations to use in the full-scale chronic tests.

Task 3--The purpose of Task 3 was to conduct life cycle chronic toxicity tests with fathead minnow to determine the effects of the test material on survival, growth, and reproduction in this fish species.

Task 4--Task 4 was reserved for special studies. The nature of these studies was to be determined for each test material after evaluating all information obtained through Task 3.

Task 5--The purpose of Task 5 was to conduct food chain transfer studies if they seemed to be warranted by the results of other studies.

MATERIALS AND EQUIPMENT

Test Materials

In this study, the substances tested were authentic LAP wastewater, synthetic LAP wastewater, 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5-trinitrobenzene (TNB), 2,4,6-trinitrobenzaldehyde (TNBA), 2,4,6-trinitrobenzonitrile (TNBN), and 4,6-dinitroanthranil (DNAN). TNT and RDX are the major organic components of LAP wastewater, and the last four compounds are phototransformation products of TNT.

We obtained TNT and RDX from SRI's explosive storage depot in Tracy, California. The TNT was purchased for another project from E.I. DuPont de Nemours and Company several years before this study began and was stored as a dry powder. The RDX was originally obtained from the Holston Army Ammunition Plant, Kingsport, Tennessee, as a powder under water. Spanggord and coworkers (1978) analyzed both compounds and found them to be at least 99.5 percent pure. The compound containers did not bear SRI identification numbers or the manufacturer's lot numbers.

We purchased TNBA from ICN-K and K Laboratories, Inc., Plainview, New York. TNB, TNBN, and DNAN were synthesized at SRI using the methods of Gilman (1941), Sitzman and Dacons (1973), and Splitter and Calvin (1955), respectively. These methods are described by Spanggord et al. (1978). These compounds were also at least 99.5 percent pure by analysis.

We obtained methyl or ring labeled ^{14}C TNT from the Midwest Research Institute, Kansas City, Missouri. The compound was manufactured by Pathfinder Laboratories, Inc. and bore lot number 61109. Uniformly labeled ^{14}C-RDX was received from E.G. and G. Bionomics, Wareham, Massachusetts. It was originally obtained from New England Nuclear Corp. and bore lot number 846-216. Both compounds came from stock purchased by these organizations to perform other studies for USAMRDC.

Authentic untreated LAP wastewater was collected from the Joliet Army Ammunition Plant, Joliet, Illinois, and shipped to SRI in polyethylene-lined 55-gallon steel drums.

Test Organisms

Four species each of freshwater fish, invertebrates, and algae were used. These are:

Pimephales promelas (fathead minnow)
Lepomis machrochirus (bluegill sunfish)

Salmo gairdnerii (rainbow trout)
Ictalurus punctatus (channel catfish)
Daphnia magna (water flea)
Hyalella azteca (scud)
Tanytarsus dissimilis (midge)
Lumbriculus variegatus (worm)
Selenastrum capricornutum (green algae)
Microcystis aeruginosa (bluegreen alga)
Anabaena flos-aquae (bluegreen alga)
Navicula pelliculosa (diatom)

The fathead minnow (all life stages), D. magna, H. azteca, L. variegatus, and T. dissimilis were from SRI's breeding colonies. We purchased the channel catfish from the Leong Catfish Farm, Modesto, California; the bluegill from the Alex Fish Company, San Rafael, California; and the rainbow trout from the Mount Lassen Trout Farm, Red Bluff, California.

N. pelliculosa was obtained from the Culture Collection of Algae, University of Texas at Houston. S. capricornutum, M. aeruginosa, and A. flos-aquae were obtained from the EPA National Eutrophication Research Program, Environmental Research Laboratory, Corvallis, Oregon.

We reared all of the animals used in the study in flowing dechlorinated tap water. For at least two weeks before being used in a test, the trout were kept at 12°C and the bluegills and catfish were kept at 20°C. Except for the minnow eggs and the 2- and 7-day-old fry used in the early life stage acute toxicity tests, all minnows were reared in 20°C water from the age of two weeks. Eggs used in the early life stage acute toxicity tests were obtained from our brood minnows.

For determining the effects of water quality on the toxicity of photolyzed and unphotolyzed LAP water (Phase II, Task 3) the test fish were acclimated to the extreme test temperatures (15°C and 25°C) for a 24-hour period immediately preceding each test. The fish were not acclimated to the different pH and hardness levels.

Minnows younger than 30 days were fed three times a day a diet consisting primarily of live brine shrimp nauplii (hatched from eggs from San Francisco Bay Brand Company, Newark, California) and some Clark's trout starter food (Clark's Feed Company, Salt Lake City, Utah). After 30 days of age, the minnows were weaned over two weeks to a diet consisting primarily of Clark's trout food supplemented with frozen brine shrimp (San Francisco Bay Brand Company). This diet was also provided to the other warm-water fish species. The trout were fed Clark's trout pellets exclusively. We fed an aqueous suspension of Clark's trout starter food and powdered alfalfa (purchased from a local health food store) to all of the invertebrates once a day. H. azteca was occasionally provided with freshly killed minnows. None of these foods were analyzed for chemical contaminants.

The algal species were cultured according to EPA-recommended procedures (EPA, 1971). Only algae from cultures in the growth phase were used in the study.

Diluent Water

We used dechlorinated tap water to culture, breed, rear, or maintain the test animals, prepare the algal culture medium (after distillation), and dilute the test materials to the desired concentrations. The water was dechlorinated by passing it through a series of columns, each containing 0.042 m^3 of activated carbon, which was renewed every three months by a local water purification firm (Culligan).

The laboratory tap water is a blend from the Hetch Hetchy, Calaveras, and San Antonio Reservoirs. On the average, about 75 percent of the water originates from the Hetch Hetchy reservoir, which is located in the Sierra Nevada. During the late spring and throughout the summer and fall, about 95 percent of the water comes from the Hetch Hetchy reservoir. During the winter and early spring before the snow begins to melt, the blend is composed primarily of water from the two low-elevation reservoirs.

The San Francisco Water Department (SFWD) annually analyzes the water from these reservoirs for various minerals and other constituents. Table 1 presents the average and range for each of the 43 parameters measured by the SFWD in 1969 to 1971 and in 1975 to 1978 in samples of water from the three reservoirs.

We performed a less comprehensive analysis of our dechlorinated water in 1975. The results are presented in Table 2. In 1978, we began analyzing the dechlorinated water routinely for hardness, alkalinity, acidity, pH, conductivity, and residual chlorine. Table 3 presents the average, standard deviation, and range for each of these parameters.

Over the seven years that our aquatic toxicology facility has been in operation, our dechlorinated tap water has been satisfactory for rearing and maintaining a variety of aquatic animals. However, during Phases I-III of this study we experienced intermittent problems with unacceptably high control mortality (>20 percent) during tests on daphnids. When this occurred, we repeated the tests until acceptable results were obtained. The problem was later determined to be caused by seasonal fluctuations in the hardness of the diluent water. Depending on the mix obtained from the three storage reservoirs, hardness dropped to levels as low as 15 mg/L (as $CaCO_3$). These periods in which the laboratory received very soft water were found to correspond to the same periods where we observed poor daphnid survival. During Phase IV, magnesium, calcium, and potassium salts were added to the diluent water to maintain the hardness at 50-100 mg/L.

Table 1. WATER QUALITY CHARACTERISTICS OF WATER FROM HETCH HETCHY, SAN ANTONIO, AND CALAVERAS RESERVOIRS

Measured Parameter	Hetch Hetchy			San Antonio			Calaveras	
	Mean[a]	Range	N[b]	Mean[a]	Range	N[b]	Mean[b]	Range
Calcium	1.08	0.3-1.6	7	31.7	23.4-50.5	4	29.3	25.7-34.5
Magnesium	0.42	0.0-1.8	7	12.0	8.6-15.1	4	10.4	7.3-13.1
Sodium	0.89	0.5-1.3	7	20.9	15.5-30.0	4	9.7	8.5-11.0
Potassium	0.34	0.2-0.6	7	2.2	2.0-2.4	4	1.6	0.9-2
Bicarbonate	6.0	2.6-9.2	7	131.6	106.6-146.4	4	114	89-146.4
Carbonate	0.0	0.0-<0.5	7	3.6	0-9.6	4	0.4	0-1.1
Carbonic Acid	2.25	1.9-2.6	2	43	0-86	2	72	-
Chloride	0.09	0.1-0.5	7	29.2	13.5-52.1	4	9.5	8-10.5
Sulfate	0.51	0.25-1.3	7	26.4	21.8-31.9	4	22.5	18.0-28.3
Fluoride	0.008	0.02-0.03	6	0.098	0-0.17	4	0.1	0.1-0.15
Aluminum	0.02	0.01-0.05	7	0.035	0.01-0.06	4	0.04	0.01-0.07
Arsenic	0.02	<0.001-0.037	2	<0.01	-	4	<0.01	-
Barium	<0.5	-	7	<0.5	-	4	<0.5	-
Cadmium	<0.002	-	7	<0.002	-	4	<0.002	-
Chromium	<0.01	-	7	<0.01	-	4	<0.005	-
Copper	0.005	0.0-0.01	4	0.5	<0.01-0.01	4	0.045	<0.01-0.06
Iron	0.06	0.005-0.12	5	0.08	<0.01-0.14	3	0.25	<0.01-0.25
Lead	<0.05	-	7	<0.05	-	4	<0.02	-
Manganese	<0.02	-	7	<0.02	-	4	<0.01	-
Selenium	<0.01	-	6	<0.01	-	4	<0.01	-
Silver	<0.03	-	7	<0.06	-	4	<0.005	-
Zinc	0.04	<0.01-0.06	3	0.016	0.009-0.03	3	0.012	0.003-0.02
Silica	3.5	0.2-6.4	7	3.7	0.2-7.0	4	7.9	3-10.5
Ammonia	0.03	0.02-0.05	5	0.15	0.02-0.214	2	0.115	0.1-0.13
Ammonia (free)	0.05	-	7	0.05	-	4	0.03	0.02-0.04
Boron	0.03	0.006-0.07	7	0.17	0.005-0.25	4	0.075	0.025-0.13
Cyanide	0.01	-	6	0.01	-	3	0.001	-
Nitrate	0.5	-	7	1.3	0.05-2.9	3	2.4	0.05-2.4
Nitrite	-	0.001-0.004	7	0.014	0.001-0.014	1	0.003	0.001-0.003
Phosphate	0.01	0-0.025	3	0.11		3	0.05	0.04-0.08
Tannins & Lignins	0.06	0.05-0.1	4	0.075	0.01-0.25	2	0.05	0.05-0.05
Dissolved oxygen	8.6	7.6-10.3	6	7.8	0.05-0.1	3	9.5	9.1-10.1
Total apparent ABS	0.05	-	7	0.05	5.9-9.0	4	0.05	-
Hardness (as $CaCO_3$)	3.6	3.0-4.6	7	128.4	-	4	116	99.0-130
Alkalinity (as $CaCO_3$)	5.1	2.6-7.5	7	107.5	94-163	4	98.8	87.3-120
Total solids	11.0	6.9-17	7	206.8	71-143	4	162.3	155-170
Conductivity (umhos/cm)	14.3	8-20	7	334.8	196-216	4	262.3	252-283
pH (units)	7.1	7.0-7.2	7	8.1	259-368	4	8.1	7.9-8.2
Turbidity (units)	0.89	0.11-4	7	2.4	7.6-8.3	4	2.4	0.3-6.4
Color (units)	1.6	0-5	7	5.0	0.1-6	4	23.3	0-50
C chloroform extract (ug/L)	-	-	7	85.6	0-10	1	-	-
Radioactivity (uci/g)								
Alpha	0.43	0.3-0.6	3	0.6	-	1	-	-
Beta	1.8	0.6-3	2	7	-	1	-	-

[a] mg/L except where marked otherwise.
[b] Number of years the specified parameter was measured.

Table 2. CHEMICAL ANALYSIS OF SRI DECHLORINATED TAP WATER

Analysis	Concentration (mg/L)
Calcium (as Ca)	8.4
Magnesium (as Mg)	2.5
Potassium (as K)	0.40
Sulfate (as SO_4)	9.2
Nitrate (as NO_3-N)	<0.005
Nitrite (as NO_2-N)	0.001
Free ammonia	0.060
Organic ammonia	0.375
Phenol	<0.001
Residual chlorine	<0.003
Chloride	4.04
Fluoride	0.30
Cyanide	<0.01
Iron	0.08
Copper	0.0041
Zinc	0.0026
Cadmium	0.0012
Chromium	0.008
Nickel	<0.050
Lead	0.0007
Total alkalinity (as $CaCO_3$)	23.3
Total hardness (as $CaCO_3$)	31.2
Total dissolved solids	48.0

Table 3. MEAN, RANGE, AND STANDARD DEVIATION (S.D.) OF WATER QUALITY PARAMETERS MEASURED ROUTINELY IN SRI'S DECHLORINATED TAP WATER (Period: May 1978 to May 1979)

Parameter	Units	Mean	S.D.	Range	Number of Analyses
Hardness	mg/L $CaCO_3$	33.8	19	11-88	45
Alkalinity	mg/L $CaCO_3$	38	20	15-90	45
Acidity	mg/L $CaCO_3$	8.0	4.6	3-20	38
pH	—	7.7	0.35	6.7-8.5	45
Conductivity	μmhos/cm	81	50	26-210	45
Total chlorine	μg/L	2.2	0.95	0.3-4.4	20

Temperature Control Equipment

To maintain the test solutions used in the static tests with the warm-water fish and the invertebrates at the desired temperatures, we partially submerged the test vessels (jars or beakers) in water baths equipped with thermostatically controlled heating elements. The temperature control system for each water bath was composed of a mercury contact thermometer (Braun), an SRI-built relay, and a 2000-W, stainless steel immersion heater. To circulate the water in each bath, we used a centrifugal water pump. The water bath used to conduct static toxicity tests with rainbow trout was equipped with a 9000-BTU water chiller in addition to the heating system. The chilling and heating systems were used together to maintain a bath temperature of 12 ± 1°C.

In the flow-through tests, we used the same kind of heating system that we used in the static tests, but the system was attached to a headbox where the water was heated and aerated before being delivered to the toxicant diluting system and the test tanks. In the flow-through tests with rainbow trout, we delivered chilled water to the headbox at a temperature slightly below 12°C and set the thermoregulator in the heating system to maintain the temperature at 12 ± 1°C. To chill the water, we used a 120,000-BTU water chiller capable of supplying about 76 liters per minute of water at a temperture of about 8°C.

To minimize temperature fluctuations of the static and flowing test solutions and to maintain the desired temperature for the tests with algae, we equipped each testing room with an air temperature control system and set the thermostat to keep the air temperature at the temperature desired for the test(s) being conducted in each room.

Toxicant Dilution Equipment

In the flow-through acute toxicity tests, we used a toxicant dilution system developed at SRI for conducting short-term tests. The system

was composed of two constant-head reservoirs, one located above the other. The upper reservoir was used to deliver toxicant stock solutions and measured about 20 x 12 x 180 cm (H x W x L). The bottom reservoir was used to deliver water and measured about 20 x 15 x 360 cm (H x W x L).

The water reservoir was equipped with 24 adjustable flow meters capable of measuring flows up to 300 mL per minute. Water was pumped to this reservoir from a secondary reservoir equipped with temperature-control and aeration devices and connected to the laboratory water supply through a float valve. Excess water in the primary reservoir returned to the secondary reservoir by gravity. The toxicant stock solution was recirculated by pump between the toxicant reservoir and a 55-gallon polyethylene-lined steel drum in which the toxicant stock was prepared. The toxicant was metered by Teflon capillary tubes; the delivery rate was adjusted by increasing or decreasing the vertical distance of the distal end of the tube from the level of the liquid in the toxicant reservoir.

Toxicant stock solution and water were delivered to a mixing chamber that divided the total volume equally between two exposure chambers. After the flows of toxicant and water necessary to obtain each desired toxicant concentration were calculated, the flows were set, using a graduated cylinder and stopwatch.

Other Equipment

Other equipment associated with performing the toxicity tests were:

- Shaker table (Labline, Model No. 3590). We used two of these rotary-action tables to conduct the algal assays. Each was equipped with a special wooden platform, which was painted white to reflect light and divided into 55 10 x 10 cm^2 sections with wooden strips. Each section accommodated one 500-mL culture flask.

- Coulter Particle Counter (Model ZBI). The particle counter was used to count algal cells in the algal assays. It was equipped with a mean cell volume computer, which we used to estimate the weight (biomass) of the algal cells.

- Spectrophotometer (Bausch and Lomb, Spectronic 20). We used the spectrophotometer to determine the concentration of chlorophyll *a* in the algal cells.

- Blender (Oster, 10-speed, Cycle Blend). The blender was used to separate the cells of the filamentous and sheet-forming algal species before counting.

- Liquid scintillation counter (Searle, Analytic Mark III). We used the scintillation counter in the bioconcentration tests to determine the concentration of ^{14}C-labelled TNT, RDX, and DNT in the exposure medium and organisms.

- Photoreactor (SRI-built). Figure 1 is a diagram of the flow-through photoreactor that we used to prepare photolyzed LAP wastewater and other photolyzed test materials in all phases of the study except Phase I. The system consisted of a quartz immersion well containing a 1200-W, medium-pressure mercury lamp (Hanovia), a Pyrex coil surrounding the well, and a quartz cooling jacket lined with aluminum foil. The Pyrex coil was used to filter out wavelengths below 294 nm and allow wavelengths above 294 nm, which approximate sunlight, to enter the coil. Pressurized air was used to cool the lamp, and water was used to cool the reactor and coil. The material to be photolyzed was introduced at the top of the Pyrex coil and pumped through it, using a Masterflex pump with Viton tubing so that any precipitate would be swept clear of the glass surfaces. For large-scale production of photolyzed material, we used a series of four reactors. In Phase I, we used a batch reactor, which was composed of an aluminum foil-lined Pyrex vessel to hold the material to be photolyzed and a Pyrex well (containing the Hanovia lamp) immersed in the aluminum-lined vessel.

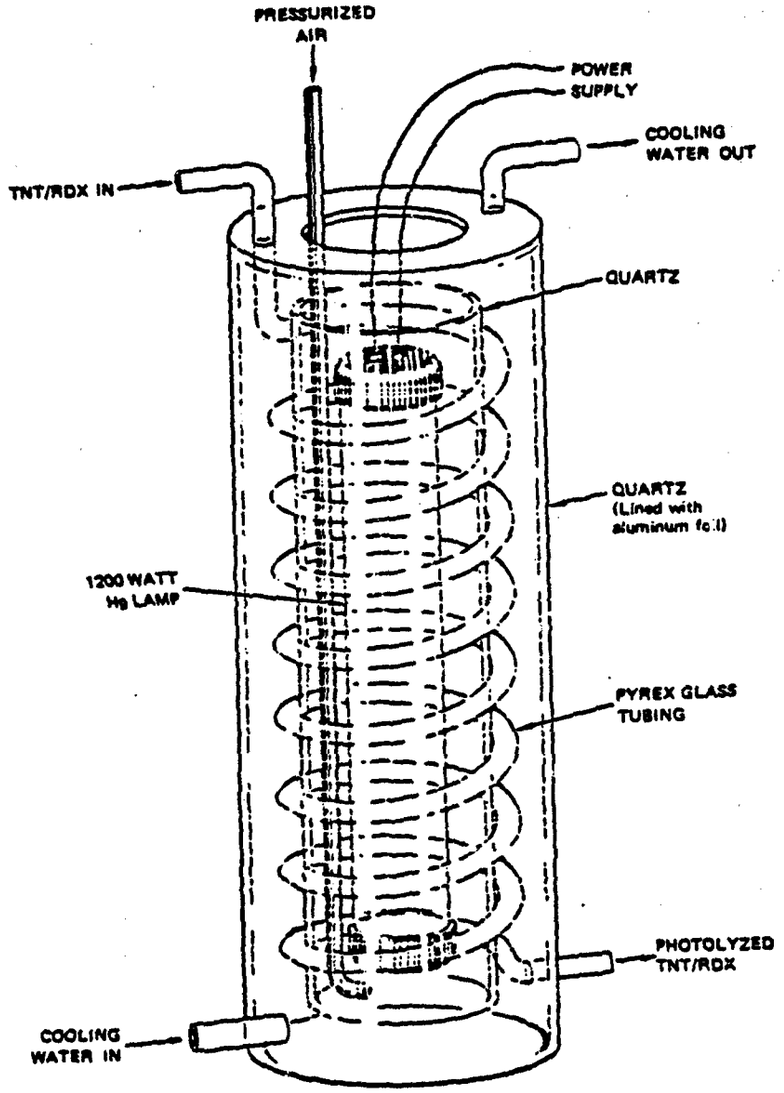

FIGURE 1 DIAGRAM OF FLOW-THROUGH PHOTOLYTIC REACTOR

METHODS

Toxicity Testing Methods

Each toxicity test entailed exposing groups of organisms of the same species to different concentrations of the test material for several days, determining the number of individuals exhibiting a preselected response to each concentration, and estimating the concentration that caused 50 percent of the organisms to respond. In most of the tests, we used six treatments, one of which was the control. In a few tests, fewer than six treatments were used; in other tests, up to nine treatments were used. The concentrations of the test materials were usually logarithmically spaced; however, an arithmetic series of concentrations was used whenever we believed that it would provide better data. The tests in Phases I and II were performed under static conditions; those in Phase III were performed under flow-through conditions.

In tests conducted with rainbow trout, we attempted to maintain the water temperature at 12 $\pm$ 1°C. The desired test temperature for all other animal species was 20 $\pm$ 1°C. For the algae, we used a test temperature of 24 $\pm$ 1°C.

Dechlorinated tap water was used in all of the tests except those conducted with algae. For the algal assays, we used a nutrient medium made from distilled tap water and selected inorganic salts.

The manual "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (EPA, 1975) was used as a guide in conducting the tests with the animal species. We performed the algal assays as described in "Algal Assay Procedure: Bottle Test" (EPA, 1971). Fish and invertebrate tests were generally repeated if control mortality exceeded 10 and 20 percent, respectively.

Static Acute Toxicity Tests. Normally, we exposed the fish species in 19-liter, wide-mouth bottles or 19-liter glass aquaria containing 15 liters of test solution. In the tests with the early life stages of the minnow, the embryos were exposed in glass or plastic (polyvinyl chloride) cylinders measuring 5 cm in diameter and 15 cm long, with one end covered with nylon screen (215-μmesh). The cylinders were suspended from a fish egg-rocking device (Mount, 1968) in 2 liters of test solution in 3.5-liter animal jars. The 2- and 7-day-old minnow fry were exposed in 250-mL glass beakers containing 200 mL of test solution. The 30- and 60-day-old fry were exposed in 3.5-liter jars containing 3 liters of test solution. All of the invertebrates were exposed in 250-mL glass beakers containing 200 mL of test solution.

We used 10 fish and 10 or 20 invertebrates per treatment. The fish were distributed in the test containers in a manner appropriate to maintaining a loading factor of $\leq$1.0 gm of fish per liter of toxicant. Twice

as many animals were used whenever the treatments were duplicated. In Task 2 of Phase I, some of the tests were of the range-finding type because only a limited amount of test material was available. In the range-finding tests, we used two or three fish or five invertebrates per treatment.

The exposure period for all fish species, including the early life stages of the minnow, was 96 hours. However, in the tests with minnow embryos we extended the exposure time to 144 or 168 hours to obtain data on egg hatching success as well as on embryo mortality. All invertebrates were exposed for 48 hours. The invertebrates and the early life stages of the minnow (embryos excepted) were fed up to the day of testing, but were not fed during the test. In tests with fish older than 60 days, food was withheld, beginning two days before the test.

We did not aerate the test solutions unless the dissolved oxygen level decreased to below 60 percent of saturation during the first 48 hours of exposure or 40 percent during the last 48 hours of exposure. If this level was reached in any of the chambers in a test or if it appeared that the dissolved oxygen concentration would decline to below that level, we delivered oil-free compressed air--through 1-mL disposable pipettes--to all of the exposure chambers in the test until the test was terminated.

The measured response for fish was death. We also determined the effect of the test materials on the number of minnow eggs that hatched. For the invertebrates, we also used death (defined by complete lack of movement and no response to prodding) as the response parameter. Dead fish (or eggs) were removed from the exposure chambers every 24 hours and counted. Motile invertebrates were counted only on termination of the test.

We determined the dissolved oxygen concentration of the test solutions daily during each test and the pH and temperature of the solutions at the beginning and end of the test. We did not determine the concentration of the toxicant in the test solutions, but calculated it after analyzing the toxicant stock solution.

Flow-Through Acute Toxicity Tests. Flow-through acute toxicity tests were used only in Phase III. In these tests, the exposure chambers were 19-liter glass aquaria. Each was equipped at one end with a standpipe drain, which was set to retain 15 liters of test solution. We evaluated each treatment in duplicate, using two exposure chambers and delivered the test solutions to the chambers from the SRI-designed toxicant diluting system (see Materials and Equipment). The fish were exposed directly in the chambers; D. magna and L. variegatus were exposed in egg cups suspended in the chambers from an egg-rocking device. We used 20 fish per chamber (40 per treatment), 10 lumbriculid worms (L. variegatus) per cup (20 per treatment), and 15 daphnids per cup (30 per treatment). Each test was terminated after 14 days of exposure or when no additional deaths occurred in any of the treatment groups for one (D. magna) or two (fish and L. variegatus) consecutive days between the 4th and 14th day.

We monitored the toxicant concentration in all exposure chambers during each test. Before initiation of a test, we prepared the toxicant stock solution and analyzed it for the toxicant. Then we set the toxicant stock and water flows to obtain the desired test concentrations and a total flow of 100 mL per minute to each exposure chamber. Before the tests were begun, the test solutions were analyzed and any necessary flow adjustment was made. After beginning a test, we determined the concentration of toxicant in samples of water collected from each exposure chamber every three to four days and whenever a fresh batch of stock solution was used. Measured chemical concentrations were used in the statistical analysis of the dose responses.

During each test, we determined the pH of all the test solutions at least twice. Dissolved oxygen levels and water temperature were determined three times a week.

Algal Assays. The toxicity of the test materials to algae was determined by the method described in "Algal Assay Procedure: Bottle Test" (EPA, 1971). At first, we determined the response of the algae by measuring three parameters; cell concentration (number of algal cells/milliliter of test solution), biomass, and chlorophyll a concentration. Cell concentration and mean cell volume were determined with the Coulter electronic particle counter. Biomass was determined indirectly by multiplying cell concentration by mean cell volume. This procedure for determining biomass is not described in the EPA algal assay manual; however, it was presented at the EPA algal assay workshop held in Corvallis, Oregon in 1975. To quantify chlorophyll a, we used the method of Shoaf and Lium (1976), who found dimethylsulfoxide (DMSO) to be a better chlorophyll extractant than acetone (which is recommended in the EPA manual) and who used a spectrophotometer rather than a fluorometer to determine the concentration of chlorophyll a.

We used the culture medium described in the aforementioned EPA manual to prepare stock solutions of the test materials and to dilute the stock to the desired test concentrations. Before use, the medium was sterilized by passing it through a 0.45- μfilter.

In each test, we used six treatment levels (including a control) and at least three cultures (replicates) per treatment. The algae were exposed to the test material in 500-mL Erlenmeyer flasks sealed with foam plugs and containing 100 mL of test solution. Exposure was initiated by inoculating each flask with 10,000 cells of S. capricornutum or 50,000 cells of A. flos-aquae, M. aeruginosa, or N. pelliculosa. During the assay, the cultures were maintained at a room temperature of 24 + 1°C on a rotating shaker table (110 rpm) under "cool-white" fluorescent lights. The light intensity at the table surface was approximately 4300 lumen/m^2 for S. capricornutum and N. pelliculosa and 2150 lumen/m^2 for the other two species. The intensities were determined with a photographic light meter.

Before counting A. flos-aquae and N. pelliculosa with the particle counter, we separated the cells by homogenizing the cultures at the

highest speed setting on the Oster blender. A. flos-aquae was homogenized for three minutes; N. pelliculosa was homogenized for one minute, inspected under a microscope, and homogenized for another minute if the first minute of processing did not fully separate the cells. The other two algal species were counted directly after their removal from the culture flasks.

We encountered several problems that led to modification in the sampling schedule and the deletion of two response parameters. Initially, we determined the concentration of the algal cells in each flask every two days; however, A. flos-aquae and N. pelliculosa did not grow well with repeated homogenization, so we reduced the frequency to the 4th and 14th days of the tests for all four species. Even at that frequency, A. flos-aquae and N. pelliculosa showed reduced growth after homogenization, so the cell concentration for these two species was determined only on the 14th (last) day of the test.

After performing the tests on TNT and evaluating the data, we decided that biomass and chlorophyll a concentration were not useful parameters and chose to measure cell concentration only. Data from tests with S. capricornutum and A. flos-aquae (Table 4) support our decision. Although we observed a decline in cell concentration with increasing TNT levels, the mean cell volumes were relatively constant. Because relative biomass is the product of cell concentration and mean cell volume, relative biomass values calculated from the data in Table 4 would reflect only changes in the cell concentrations.

The major reason for rejecting chlorophyll a as a response parameter was interference apparently caused by photolyzed TNT. We found that after TNT was exposed to the fluorescent lights required to culture the algae, a substance or substances appeared that strongly absorbed at 663 nm--the wavelength of maximum absorbance by chlorophyll a. Analysis of the test solutions for TNT showed a decline of about 28 to 74 percent in the initial TNT concentrations over 14 days. That the decline was caused by photolysis was evidenced by the appearance of a reddish color in the flasks containing TNT.

Exploratory Bioconcentration Tests. The bioconcentration tests were performed with 14C-labeled TNT and RDX, both alone and together. In each test, the exposure period was 96 hours. The amount of radioactivity in the organisms was determined only at the end of the exposure period. The tests were conducted under static conditions at 20°C with the animal species and 24°C with the algae.

The tests on TNT and RDX alone were performed with the L. macrochirus (bluegill), L. variegatus (worm), D. magna (water flea), and S. capricornutum (green algae). Tests on the TNT-RDX mixture were performed only with the bluegill. We used three bluegills in 10 liters, 50 worms or 100 adult daphnids in two liters, and started with about 10,000 algal cells in 100 mL of test solution.

For the tests with the animal species, we prepared 30 liters each of radiolabeled solutions using dechlorinated tap water. We received the

Table 4. EFFECT OF TNT ON CELL CONCENTRATION, MEAN CELL VOLUME AND CHLOROPHYLL *a* CONCENTRATION IN TWO SPECIES OF ALGAE AFTER 14 DAYS OF EXPOSURE

Nominal TNT Concentration (mg/L)	S. capricornutum			A. flos-aquae		
	Cell Concentration (cells/mL)	Mean Cell Volume (nm^3)	Chlorophyll *a* Concentration (mg/L)	Cell Concentration (cells/mL)	Mean Cell Volume (nm^3)	Chlorophyll *a* Concentration (mg/L)
0	4,617,120	80	1.75	2,445,440	75	5.84
2.5	2,823,280	95	2.97	2,109,093	68	5.24
5	1,667,280	87	2.87	1,933,307	85	3.28
10	1,393,920	72	3.77	1,125,613	83	3.28
20	17,600	83	6.47	48,240	76	5.14
41	18,320	85	5.14	40,773	66	4.89
82	16,640	79	4.60	31,147	73	4.30

labeled RDX dissolved in DMSO, so we also dissolved the labeled TNT in DMSO before preparing the test solutions. The TNT solution contained 0.5 mg of TNT and 0.2 μCi of radioactivity per liter. The RDX solution contained 0.3 mg of RDX and 0.2 Ci of radioactivity per liter. To prepare the TNT-RDX mixture, we combined equal volumes of the TNT and RDX solutions. The ratio of TNT to RDX in this mixture was 1.6:1, the same as in LAP wastewater. For S. capricornutum, we prepared the TNT and RDX solutions in smaller volumes to the same concentrations and levels of radioactivity, using algal culture medium.

After 96 hours of exposure, we collected the algae, daphnids, and worms on Whatman GF/F glass fiber filters, removed them from the filters, and weighed them. From each fish, we removed a sample of muscle tissue and the viscera (gills and kidneys excluded) and weighed the tissues. All of the samples were solubilized in Unisol (Isolab, Inc.) and 1 mL of the solubilized material was transferred to a cocktail containing 1 mL of water-free methanol, 0.25 mL of 2-ethylhexanoic acid, and 10 mL of Aquasol (Packard Instrument Co.). The mixtures were counted for 10 minutes in a liquid scintillation counter equipped with an automatic background subtractor. To determine the radioactivity of the test solutions, we collected samples at the beginning of each test and added 0.5 mL of a sample to 10 mL of Aquasol before analyzing it for radioactivity.

We counted two subsamples of each sample twice and averaged the counts. The counting efficiency was determined using an internal standard. Counts per minute (cpm) were converted to disintegrations per minute (dpm) by dividing the cpm by the counting efficiency. The bioconcentration factor (BCF) was calculated by dividing the number of dpm found in the tissues by the number found in the test solution.

Preparation of Stock Solutions of the Test Materials

We prepared the test solutions of all of the test materials by diluting a concentrated stock solution of each material to the desired test concentrations. The stock solutions of all materials except authentic LAP wastewater were prepared by dissolving the materials directly in an appropriate aqueous medium, which was dechlorinated tap water for the tests with the animal species and a nutrient formulation for the algae. The stock solution of authentic LAP wastewater was the wastewater as received from the Joliet ammunition plant.

Stock solutions of different synthetic LAP wastewaters were prepared by combining chemically analyzed solutions of TNT and RDX in the proper proportions to obtain solutions containing ratios of 1.6:1, 1:1, 1:3, or 3:1 TNT to RDX. We found it impossible to consistently obtain these ratios by adding undissolved TNT and RDX to the same vessel of water.

After preparing each stock solution, we filtered the solution through a 5-μm cartridge filter and analyzed the filtrate for the test material. We did not filter authentic LAP wastewater. The chemical analytical results were used as a guide in diluting the stock to the desired test concentrations.

We prepared photolyzed synthetic and authentic LAP wastewater and solutions of TNT by irradiating unphotolyzed stock solutions of these materials in either the batch reactor (Phase I only) or the flow-through photolytic reactor (both are described in the Materials and Equipment section). During the irradiation process, we periodically withdrew samples of liquid from the reactor, analyzed them for TNT (and RDX when appropriate), and stopped the reactor when the desired degree of photolysis had occurred.

Preparation of Aqueous and Benzene Fractions of LAP Wastewater and Photolyzed TNT

To prepare aqueous and benzene fractions of LAP wastewater and a 50 percent photolyzed solution of TNT for acute toxicity evaluation in Task 2 of Phase I, we mixed 5 liters of the wastewater or 8 liters of the photolyzed TNT solution with an equal volume of benzene in a separatory funnel. After shaking the mixture, we separated the aqueous and benzene layers. Photolyzed TNT was prepared by batchirradiating a solution of TNT containing 147 mg/L of TNT until the TNT concentration was reduced to 75 mg/L. Irradiation was performed at pH 7.

After separating the two fractions, we lyophilized 175 mL of the benzene fraction and weighed the residue to determine the concentration of total dissolved solids (TDS). Another aliquot (20 mL) of the benzene fraction was rotary-evaporated to dryness, dissolved in 1 mL of dichloromethane, and analyzed for TNT by gas chromotography, using m-dinitrobenzene as the internal standard. The rest of the fraction was rotary-evaporated to dryness, lyophilized, weighed, and dissolved in a small volume of acetone. The acetone stock solution was diluted with dechlorinated water and evaluated for toxicity.

To remove benzene from the aqueous fraction, we heated the fraction to 60°C under vacuum while aerating it with nitrogen gas. An aliquot of the heated liquid was periodically removed and analyzed by gas chromatography for benzene, using phenol as the internal standard. After benzene was no longer detectable, we lyophilized a 40-mL aliquot and weighed the residue to determine the concentration of TDS. Another 20-mL aliquot was extracted with ether. The ether extract was rotary-evaporated to dryness, adjusted to a final volume of 1 mL with dichloromethane, and analyzed for TNT in the manner used for the benzene fraction. The remaining portion of the fraction was evaluated for acute toxicity without further processing.

pH Adjustments

In Tasks 1 and 2 of Phase I, we used 0.1N HCL or 5N NaOH to adjust the pH of the test materials to 5, 7, or 9.4. In Task 3 of Phase II, we used a $NaOH-KH_2PO_4$ buffering solution to adjust the pH of the test solutions to 6, 7, or 8.

Chemical Analytical Methods

The concentrations of TNT, RDX, TNT/RDX (LAP water), 1,3,5-trinitrobenzene (TNB), 2,4,6-trinitrobenzaldehyde (TNBA), and 4,6-dinitroanthranil

(DNA) in the test and/or stock solutions were determined by high-pressure liquid chromatography (HPLC). The instrument (Spectra-Physics, Model 3500B) was equipped with a 3.9 mm by 30 cm C_{18} - Bondapak column for the analysis of TNT, RDX, TNT/RDX, and DNAN and with a 3.9 mm x 30 cm Spherisorb ODS column for the analysis of TNB and TNBA. Various mixtures of methanol and water were used as eluting solvents. All analyses were performed by direct injection of the aqueous samples into the chromatograph. Table 5 presents the HPLC parameters for those toxicants.

The concentration of TNBN in the solutions was analyzed with a gas chromatograph (Hewlett-Packard, Model 5711) equipped with a 1.8 m x 2 mm glass column packed with 10 percent DC-200 on Gas Chrom Q and a flame ionization detector. The nitrogen gas flow rate was 25 mL/min, and the temperature regimen was set for 155°C for 2 minutes, then progressing to 220°C at 4°C/min. The retention times for TNBN and benzophenone (10 mL, the internal standard) were 6.82 and 4.62 min, respectively. Aqueous samples containing the compound were extracted with ethyl acetate (2 x 10 mL), and the extracts were combined. After the addition of benzophenone to the extract, the extract was rotary-evaporated to 5 mL, further reduced to 0.2 mL with a stream of nitrogen gas, then injected into the gas chromatograph for analysis.

Statistical Methods

Estimation of the LC50

To estimate the median lethal concentration (LC50) we used a computerized program developed at SRI and composed of several statistical methods for estimating LC50s and EC50s. For this project, we chose estimates derived only from the log-probit or binomial methods. The specific method we chose depended on the number and pattern of partial responses (i.e., >0 percent, <100 percent).

We used the binomial method when there were no partial responses or when only one incongruous response or nonresponse occurred. A single response (e.g., death) at a concentration was considered to be incongruous when no responses occurred at the next higher concentration. Also, a single nonresponse (e.g., non-death) at a concentration was considered to be incongruous when at the next lower concentration, all of the organisms responded. We also used the binomial method when other incongruous response patterns occurred (e.g., 0, 0, 100, 90, 100, and 100 percent mortality in a series of six increasingly higher test concentrations).

The binomial method is valid regardless of the form of the underlying tolerance distribution and therefore gives statistically valid, but conservative confidence intervals in all cases. It is the only appropriate method when a data set contains no partial responses. The method is a two-step process. In the first step, at each concentration level with an observed mortality of 50 percent or more, a significance level is computed for the hypothesis that the true mortality at that concentration is 50 percent or less, using only the observations at that concentration. In the second step, at each concentration level with an observed mortality of less than 50 percent, a significance level is computed for the hypothesis that the true mortality at that concentration is 50 percent or more. An

Table 5. HPLC PARAMETERS FOR ANALYSIS OF THE TEST MATERIALS

Parameter	Test Materials				
	TNT	TNT/RDX	TNB[a]	TNBA[a]	DNAN[a]
Concentration range (mg/L)	0.06-2.0	0.1-3.0 (TNT) 2.0-6.0 (RDX)	50-100	50-100	50-100
Loop Size (λ)	450	450	100	100	100
Methanol/Water (V/V)	51/40	47/53	55/45	57/43	45/55
UV Wavelength (nm)	254	254	254	254	254
Flow Rate (mL/min)	2.0	2.0	1.6	1.6	1.2
Internal Standard	3,4-Dinitro-toluene	3,4-Dinitro-toluene	Benzo-phenone	Benzo-phenone	3,5-Dinitro-toluene
Retention Time (sec)	334	178-201 (RDX) 300-377 (TNT)	118	113	415
I.S. Retention Time (sec)	465	413-539	441	349	833
Relative I.S./Response	1.4	3.8 (RDX) 1.4 (TNT)	1.1	3.1	2.1
UV Sensitivity (aufs)[c]	0.01	0.05	0.04	0.04	0.04
Accuracy (%)	±1.4	±1.2-1.5	±1.0	±1.0	±1.0
Precision (%)	±1.4	±1.0	--[b]	--[b]	--[b]
Detection Limit (mg/L)	0.04	0.04 (TNT) 0.09 (RDX)	--[b]	--[b]	--[b]

[a]TNB - Trinitrobenzene; TNBA - Trinitrobenzaldehyde; DNAN- Dinitroanthranil.

[b]Not determined.

[c]Full-scale absorbance units.

estimate of the LC50 is also provided as the geometric average of the adjacent concentrations with zero and 100 percent mortality. The 95 percent confidence interval for the LC50 is the shortest interval (with endpoints at the concentrations or at plus or minus infinity) such that at the upper endpoint and all higher concentrations, 50 percent or more of the animals have died and the significance level is 0.025 or less, and at the lower endpoint and all lower concentrations, less than 50 percent of the animals have died and the significance level is 0.025 or less.

The probit method is a parametric technique that depends on the assumption that the tolerance of the organisms to the test material follows a normal distribution. The computer routine performs the probit analysis twice—once for the concentration levels expressed in linear units and once for the concentration levels expressed in logarithmic units. In either case, Berkson's adjustment (one-half of a response at the highest concentration with no response and one-half of a nonresponse at the lowest concentration with all 100 percent response) is used when there is only one partial response.

The LC50 estimate is the maximum likelihood estimate for the mean of the tolerance distribution. The "unadjusted" confidence interval for the LC50 is derived by inverting the likelihood ratio test for determining whether any specified concentration is the LC50. A Chi Square test is computed to determine how well the estimated tolerance distribution fits the data (which are also plotted). In computing this test, adjacent concentration levels are collapsed until the expected responses (mortality and nonmortality) are everywhere greater than 2.0. Finally, if the probability of poor fit is 0.75 or greater, a heterogeneity factor is derived from the Chi Square test and the confidence interval is adjusted outward, using the heterogeneity factor.

When the probability of poor fit was given, we used the probit model (linear and logarithmic) with the smaller probability of poor fit, and the confidence interval was adjusted outward using the heterogeneity factor. When the probability of poor fit was unavailable, we used the logarithmic model and the unadjusted confidence intervals.

Differences Between LC50s

Whenever it was necessary to determine if one test material was more or less toxic than another, if one test species was more or less resistant than another to a test material, or if the toxicity of a test material was affected by some treatment or water quality parameter, we first examined the 95 percent confidence limits of the two LC50s being compared. When the 95 percent confidence limits of one of the estimates (LC50) included the other estimate, we considered the difference between the two estimates to be statistically insignificant. If the 95 percent confidence limits of the two estimates did not overlap, we considered the two estimates to be significantly different. When the 95 percent confidence limits of the two estimates overlapped but did not include either toxicity estimate, we performed Student's *t* test to determine whether the difference between the estimates was statistically significant. In all cases, we used a significance level of five percent.

Statistical Analysis of Algal Assay Data

We analyzed data from the algal assays using an outlier test recommended by EPA (1971), followed by the application of the SPSS (Statistical Package for the Social Sciences) analysis of variance package (one-way).

The outlier test (adopted by the EPA from Section 3.8 of Applied Regression Analysis Draper and Smith, 1968) was used on the control group at the 5 percent significance level. After rejecting the outliers, we computed the F value. Whenever the F test was significant at the 5 percent level, control group contrasts were examined to identify the toxicant-exposed groups that differed significantly from the controls. Because the tests for homogeneity of variance (Cochran's C and Bartlett-Box's F tests) typically indicated that the variances were unequal, we computed the contrasts using separate variance estimates. These contrasts were referred to t-tables to establish the significance level using an interpolated number of degrees of freedom. The significance levels recorded in the tables are one-sided.

Characterization of Toxic Interaction Between Two Compounds in a Mixture

To determine mathematically whether the toxicity of mixtures of TNT and RDX were more or less than the sum of the toxicities of TNT and RDX, we used a modified version of a method developed by Marking and Dawson (1975). These authors calculated the sum (S) of the contributions of two compounds (A and B) to the toxicity of a mixture of the two compounds by the equation:

$$\frac{A_m}{A_1} + \frac{B_m}{B_1} = S$$

where A_1 and B_1 are the individual LC50s of compounds A and B, and A_m and B_m are the LC50s of compounds in the mixture. The values A_m and B_m are calculated by multiplying the LC50 of the mixture by the fraction that each compound contributes to the total concentration of both compounds in the mixture.

To explain the logic of Marking and Dawson's equation, we have rewritten it as follows:

$$S = \frac{aLC50_M}{LC50_A} + \frac{bLC50_M}{LC50_B}$$

where $LC50_A$, $LC50_B$, and $LC50_M$ are the experimentally derived estimates of acute toxicity for compounds A, B, and the mixture (M), respectively, and a and b are the respective fractions of compounds A and B in the mixture.

If $LC50_A$, $LC50_B$, and the amount of A and B in M are known, the theoretical concentrations of M that would kill 50 percent of the test organisms (under the assumption that A and B were neither antagonistic nor synergistic) can be calculated. We shall call that concentration the additive LC50 of M and denote $LC50_M$*.

On the basis of acute toxicity tests, each gram of compound B is only $LC50_A/LC50_B$ as toxic as each gram of compound A. If we assume that the toxicities of A and B are additive, each gram of the mixture M should be as toxic as $a + b(LC50_A/LC50_B)$ grams of A. Thus, the additive LC50 of M is:

$$LC50_M^* = \frac{LC50_A}{a + b(LC50_A/LC50_B)}$$

By algebraic manipulation, it can be shown that:

$$S = LC50_M/LC50_M^*.$$

The statistic S ranges from zero to infinity, with a value of 1.0 denoting additivity. Because the range of S is nonsymmetric around 1.0, Dawson and Marking (1975) suggested replacing S by a corrected sum, which we shall call CS, and which these authors defined as: $CS = 1/S-1$ when $S \leq 1$ and $CS = 1 - S$ when $S > 1$.

We found little merit in this definition and redefined CS as CS = LogS. This transformation symmetrizes the range around zero. A CS value of zero indicates additivity, a CS value of -1 corresponds to $LC50_M = LC50_M^*/10$ (or synergism), and a CS value of +1 corresponds to $LC50_M = 10(LC50_M^*)$ (or antagonism). This transformation also simplifies the derivation of the confidence interval for CS.

Marking and Dawson (1975) derive the 95 percent confidence interval for CS by substituting the 95 percent confidence intervals for $LC50_A$, $LC50_B$, and $LC50_M$ into their equation for calculating S. We found this heuristic procedure to be unjustifiable because the 95 percent confidence interval for CS (i.e., Log S) can be rigorously defined. From Equation 3,

$$\text{Log } S = \text{Log } LC50_M - \text{Log } LC50_M^*$$

If the central limit theorem can be invoked (e.g., if Log $LC50_M$ and Log $LC50_M$* can be assumed to be normally distributed), then an approximate 95 percent confidence interval for the true corrected sum is:

$$\text{Log } S \pm 1.96[\text{VAR}(\text{Log } LC50_M) + \text{VAR}(\text{Log } LC50_M^*)]^{1/2}$$

where VAR denotes variance. The variance of Log $LC50_M$ can be estimated from the results of the toxicity test on the mixture. The variance for Log $LC50_M$* can be estimated by the equation:

$$\text{VAR}(\text{Log } LC50_M^*) = \left(1 - \left(\frac{aLC50_B}{aLC50_B + bLC50_A}\right)^2\right) \text{VAR}(\text{Log } LC50_B) + \left(1 - \left(\frac{bLC50_A}{aLC50_B + bLC50_A}\right)^2\right) \text{VAR}(\text{Log } LC50_A) \quad (6)$$

RESULTS AND DISCUSSION

Phase I - Preliminary Studies

Task 1 - Effect of Photoirradiation Under Different Conditions of pH on the Toxicity to Fathead Minnows of LAP Wastewater and TNT

In Task 1, we conducted nonreplicated 96-hour static acute toxicity tests with fathead minnows on LAP wastewater and solutions of TNT that had been exposed to simulated sunlight at pH 5, 7, or 9.4 and adjusted to pH 7 before testing. The objective of this task was to determine if the degree of photolysis and the pH of the test materials during irradiation had an effect on the toxicity of the test materials. The degree of photolysis of LAP wastewater and TNT was based on the percentage difference in the initial and final concentrations of TNT.

Table 6 presents the 96-hour LC50s obtained in tests with fathead minnows on LAP wastewater photolyzed at the three levels of pH. We found no statistically significant differences between the LC50s of the nonphotolyzed wastewater. This indicates that pH does not affect the toxicity of nonphotolyzed LAP wastewater.

Table 6. ACUTE TOXICITY TO FATHEAD MINNOWS OF LAP WASTEWATER EXPOSED TO ULTRAVIOLET LIGHT UNDER DIFFERENT CONDITIONS OF pH

Degree of Photolysis (%)[a]	pH	96-Hour LC50 (% Wastewater)	95% Confidence Limits
9	5	2.7	2.5-3.0
49	5	3.3[c]	2.9-3.9
0	7	2.7	2.3-3.2
49	7	3.7[c]	3.2-4.4
100[c]	7	>9.5[c]	—[b]
0	9.4	3.2	2.8-3.8
50	9.4	4.0	3.4-4.7

[a]Percentage difference between the initial and final measured concentration of TNT in the wastewater.

[b]Not determined.

[c]Test solutions aerated.

Partial photolysis (about 50 percent) of the wastewater reduced its toxicity slightly at all levels of pH. The absolute change in toxicity was small. The maximum change occurred at pH 7, where the 96-hour LC50 shifted from 2.7 percent for the nonphotolyzed wastewater to 3.7 percent for the 50 percent photolyzed wastewater (a 37 percent increase); the difference between the LC50s was statistically significant. Also statistically significant was the difference between the two LC50s at pH 5. At pH 9.4, the difference between the LC50s of 3.2 percent and 4.0 percent was not significant.

We investigated the effect of 100 percent photolysis (≥99.8% degradation of parent chemical) of LAP wastewater only at pH 7. The tested concentrations were not high enough to obtain a 96-hour LC50; however, the toxicity of the photolyzed wastewater was relatively low. The highest tested concentration (9.5 percent) killed only 10 percent of the minnows and none of the lower tested concentrations caused any observable effects.

Table 7 presents the results of similar tests on TNT. The 96-hour LC50s of the pH-adjusted, nonphotolyzed solutions increased with pH. We found no statistically significant difference between the LC50s obtained for the solutions adjusted to pH 7 and 9.4. However, the LC50 obtained for the pH 5 solution was significantly lower than either of the other LC50s. This suggests that TNT may have undergone a chemical change while it was maintained at pH 5 and that the change not only increased

Table 7. ACUTE TOXICITY TO FATHEAD MINNOWS OF TNT EXPOSED TO SIMULATED SUNLIGHT UNDER DIFFERENT CONDITIONS OF pH

Degree of Photolysis (%) [a]	pH	96-Hour LC50 (mg/L) [b]	95% Confidence Limits
0	5	1.2	0.7-1.6
47	5	>3.2	— [d]
0	7	2.1	1.8-2.5
49	7	>3.2 [c]	— [d]
100	7	52.9	47.1-75.7
0	9.4	2.4	2.0-2.9
51	9.4	>3.2	— [d]

[a] Percentage difference between the initial and final measured concentration of TNT in the wastwater.

[b] Based on the concentration of TNT before photolysis.

[c] Test solutions aerated.

[d] Not determined.

its toxicity, but was not influenced when the pH of the solution was adjusted back to 7 before testing.

In the tests on partially photolyzed (about 50 percent) TNT, we diluted the stock solutions of photolyzed TNT the same way we diluted the stock solutions of nonphotolyzed TNT. Before they were irradiated, the stock solutions of photolyzed TNT contained exactly the same amount of TNT as did the stock solutions of nonphotolyzed TNT. Thus, percentagewise, the concentration series prepared from both stocks were identical. They were not identical with respect to toxicity, however. Whereas 3.2 mg/L of nonphotolyzed TNT caused at least 50% mortality at all levels of pH, the equivalent concentration of photolyzed TNT caused less than 50 percent mortality.

Table 8 shows for partially photolyzed TNT the mortality observed at each nominal test concentration. The solution irradiated at pH 5 caused 17 percent mortality at a concentration equivalent to 3.2 mg/L of TNT, but it was not toxic at the lower concentrations. Whereas the 96-hour LC50 for nonphotolyzed TNT was 1.2 mg/L, photolyzed TNT concentrations equivalent to up to 2.4 mg/L of TNT did not kill any of the fish. The solution irradiated at pH 7 caused some mortality at all test concentrations. The concentration-mortality pattern was somewhat irregular; however, because none of the control group died, we believe that the deaths were caused by the test material. This solution was more toxic than the solutions irradiated at pH 5 and 9.4, but not as toxic as the corresponding nonirradiated solution. No deaths occurred at any of the tested concentrations of the solution irradiated at pH 9.4.

Table 8. 96-HOUR MORTALITY AMONG FATHEAD MINNOWS EXPOSED TO 50 PERCENT PHOTOLYZED TNT SOLUTIONS EXPOSED TO SIMULATED SUNLIGHT AT pH 5, 7, AND 9.4

TNT Concentration[a] (mg/L)	Mortality (%)		
	pH 5	pH 7	pH 9.4
0	0	0	0
1.0	0	10	0
1.4	0	30	0
1.8	0	10	0
2.4	0	30	0
3.2	17	40	0

[a]Nominal; based on the concentration of TNT in solution before irradiation.

We believe that these data indicate that partial photolysis of TNT at pH 5 and 9.4 reduces the toxicity of TNT; however, at pH 7, partial photolysis has little effect on the toxicity of the compound.

Complete (≥ 99.8 percent degradation of the parent chemical) phototransformation of TNT at pH 7 caused a large (25-fold) and statistically significant reduction in the toxicity of the TNT solution (Table 7).

According to Bentley and coworkers (1978), the 96-hour LC50 for RDX in fathead minnows is 5.3 mg/L, which is comparable to the value of 4.5 mg/L obtained for RDX at SRI. Both of these values are higher than the 96-hour LC50s we obtained for fathead minnows exposed to TNT (1.2 - 2.4 mg/L). Since LAP wastewater is composed primarily of TNT and RDX (Spanggord et al., 1978), we believe that the toxicity of LAP wastewater is caused primarily by TNT.

We obtained partial evidence that TNT is the controlling factor by converting the 96-hour LC50s presented for LAP wastewater in Table 6 from percent wastewater to milligrams of TNT per liter of solution. Analysis of the wastewater showed that it contained 82.2 mg/L TNT. Multiplying this value by the LC50s presented in Table 6 for nonphotolyzed wastewater and dividing the product by 100, we obtained 96-hour LC50s for TNT of 2.2, 2.2, and 2.6 mg/L, respectively, for the wastewater irradiated at pH 5, 7, and 9.4. These LC50s compare well with the 96-hour LC50s obtained for pH 7 and 9.4 TNT solutions (Table 7), but not with the LC50s obtained for the pH 5 TNT solution.

During the tests on nonphotolyzed and 50 percent photolyzed LAP wastewater and TNT, some of the fish exposed to concentrations that caused deaths developed a hemorrhagic lesion just below the dorsal fin. The lesion was internal, apparently involving the spinal column, and caused a bilateral bulge. The lesion usually appeared during the first 24 hours of exposure, and fish with the lesion usually died during the 96-hour tests. Fish without lesions also died, however. The lesion probably was caused by TNT because it did not occur in fish exposed to completely photolyzed LAP wastewater or TNT solutions.

The wastewater and TNT solutions also caused behavioral changes that included listlessness and tail-drop, progressing to severe loss of equilibrium evidenced by upside-down or sideways swimming. Before death, affected fish lay almost motionless on the bottom of the exposure chamber; their respiration was shallow and rapid, and prodding caused rapid, uncoordinated muscular movement. We observed both effects in all subsequent tests on nonphotolyzed LAP and TNT solutions.

Task 2 - Acute Toxicity of the Aqueous and Benzene Fractions of LAP Wastewater and Photolyzed TNT

Table 9 presents the results of the acute toxicity tests with the fathead minnow and D. magna on the aqueous and benzene fractions of LAP wastewater and a solution of 50 percent photolyzed TNT. We were not able to evaluate the fractions as thoroughly as we had intended because of the limited volume of test material prepared. Nevertheless, we believe that the results of the tests indicate that the benzene fractions were more toxic than the aqueous fractions.

Table 9. RELATIVE TOXICITY OF THE AQUEOUS AND BENZENE FRACTIONS OF LAP WASTEWATER AND PHOTOLYZED TNT TO THE FATHEAD MINNOW AND D. MAGNA

Test Sample	TDS[a] (mg/L)	TNT (mg/L)	Toxic Level[b] (mg/L TDS) Fathead Minnow	D. magna
LAP Wastewater				
Aqueous fraction	1150	3.7	>100	>3.7
Benzene fraction	275	78.5	4.8 (4.0-5.6)[d]	>10
Photolyzed TNT				
Aqueous fraction	207.5	12.3	20.6 (17.0-24.7)	50-100[c]
Benzene fraction	57.1	63.3	5.7 (3.2-infinity)	8-10[c]

[a]Total dissolved solids.
[b]48- or 96-hour LC50.
[c]Concentrations that bracket the 48-hour LC50.
[d]Test solutions aerated.

We interpret the results to mean that toxicity of LAP wastewater and partially photolyzed TNT is caused primarily by their nonpolar constituents. In LAP wastewater, the major nonpolar components are TNT and RDX. Except for TNT, we are not certain what other nonpolar compounds were present in the solutions of partially photolyzed TNT. Probable ones are TNB, TNBA, 2,4,6-trinitrobenzaldehyde, and DNAN. The TNT solution was irradiated at pH 7; hence, TNBN should not have been present. These compounds are probably not the only phototransformation products of TNT, thus, other nonpolar compounds could have been present.

There was a discrepancy in the relative concentrations of TDS and TNT in the benzene fraction of photolyzed TNT (see Table 9). We believe that value for TNT is reliable because we analyzed the fraction for that compound before removing the benzene. However, we believe that the value for TDS is low and that the low value was caused by the loss of volatile organic compounds when the fraction was evaporated to dryness. We believe that the TDS value for the benzene fraction of LAP wastewater may also be low for the same reason.

Task 3 - Toxicological Interaction with TNT and RDX, and Acute Toxicity of Four Phototransformation Products of TNT

Table 10 presents the 96-hour LC50s obtained in tests with fathead minnows for TNT and RDX alone and for various mixtures of TNT and RDX. It also presents, for each mixture, the interactive index, which we calculated using our modified version of the method of Marking and Dawson (1975). Our interactive index is analogous to Marking and Dawson's additive index.

Table 10. TOXICOLOGICAL INTERACTION OF TNT AND RDX

Test Material	96-Hour LC50 and 95% Confidence Limits (mg/L)	Interactive Index and 95% Confidence Limits
TNT	2.4 (2.0 to 3.2)	N.A.[a]
RDX	4.5 (3.7 to 5.4)	N.A.[a]
TNT/RDX (1:1)	5.4 (4.8 to 6.4)	0.237 (0.12 to 0.31)
TNT/RDX (1:3)	6.8 (6.1 to 7.6)	0.265 (0.18 to 0.34)
TNT/RDX (3:1)	1.9 (1.7 to 2.3)	-0.155 (-0.04 to -0.27)
TNT/RDX (1.6:1)[b]	5.9 (5.4 to 6.8)	0.34 (0.21 to 0.40)

[a]Not applicable.
[b]From Table 14.

An interactive index of zero indicates that the toxicities of two compounds in a mixture are additive. Positive indices indicate antagonism, and negative indices indicate synergism. When the 95 percent confidence limits of a positive or negative index encompass zero, we considered the toxicities of the two compounds to be additive.

All of the TNT/RDX mixtures except the 3:1 mixture had positive indices and 95 percent confidence limits. This indicates that in those mixtures, TNT and RDX acted antagonistically, causing the mixtures to be less toxic than the sum of the toxicities of the two compounds. For the 3:1 mixture, the index of its 95 percent confidence limits were negative, thus, in this case, TNT and RDX exhibited synergism.

The extent of antagonism and synergism was relatively minor. An index of -1 indicates that the observed LC50 of a mixture is 10 times less than the theoretical additive LC50 of the mixture, and an index of +1 indicates that the observed LC50 is 10 times greater than the theoretical additive LC50. The interactive indices for the mixtures tested ranged between -0.155 (1.43 times less than the theoretical additive LC50) and 0.34 (2.19 times more than the theoretical additive LC50).

Table 11 presents the 96-hour LC50s and 48-hour LC50s obtained from fathead minnows and D. magna, respectively, for TNB, TNBA, TNBN, and DNAN, which are phototransformation products of TNT. For comparison, we have listed the 48-hour LC50 and 96-hour LC50 for TNT.

The 96-hour LC50s and 48-hour LC50s for all of the phototransformation products were significantly lower than those for TNT. DNAN, with a 96-hour LC50 and 48-hour LC50 of 0.16 and 0.34 mg/L, respectively, was significantly more toxic than the other phototransformation products.

Table 11. ACUTE TOXICITY TO FATHEAD MINNOWS AND D. MAGNA OF TNT AND FOUR OF ITS PHOTOTRANSFORMATION PRODUCTS

Compound	96-hour LC50 in Minnows (mg/L)		48-hour LC50 in D. magna (mg/L)	
TNT	2.9	(2.6 -3.2)[a,b]	11.7	(10.9 -12.6)[c]
TNB	1.1	(1.0 -1.2)	2.7	(2.4 - 3.1)
TNBA	1.0	(0.9 -1.2)	1.5	(0.9 - 2.7)
TNBN	2.0	(1.6 -2.3)[b]	1.0	(0.8 - 1.2)
DNAN	0.16	(0.12-1.0)[b]	0.34	(0.24- 1.0)

[a] 96-hour LC50 from Table 14.
[b] Test solutions aerated.
[c] 48-hour LC50 from Table 15.

Initially, we found it difficult to believe that the phototransformation products of TNT could be more toxic than TNT because we had relatively conclusive evidence that photoirradiation reduced the toxicity of TNT. The inconsistency was resolved after reviewing the work of Burlinson and coworkers (1973); the maximum yields of TNB, TNBA, TNBN, and DNAN that they obtained by photoirradiating TNT were 1, 10, 4, and 4 percent, respectively, of the initial amount of TNT.[1] Using these yields, we calculated that if 1.9 mg/L TNT (the 96-hour LC50 for TNT) were completely photolyzed, the yields of TNB, TNBA, TNBN, and DNAN would be 0.029, 0.29, 0.116, and 0.116 mg/L, respectively; and if 11.7 mg/L TNT (the 48-hour LC50 for TNT) were completely photolyzed, the respective yields would be 0.117, 1.2, 0.47, and 0.47 mg/L. These yields indicate that except for DNAN, these compounds were probably not present at lethal concentrations in the solutions of photolyzed TNT that we tested. TNBA, TNBN, and DNAN are photolabile; thus, it is possible that their concentrations in the tested photolyzed solutions were even less than calculated above.

Phase II - Studies on Synthetic LAP Wastewater (LAP Water) and Further Studies on TNT

Task 1 - Standardization of Photolyzed LAP Water

Table 12 presents the 48-hour LC50s obtained with D. magna in quadruplicate toxicity tests on LAP water irradiated at five flow rates in the flow-through photolytic reactor. The final concentrations of TNT

[1] Note that the systems used by Burlinson et al., and SRI were similar except for the light intensities. The system used by SRI utilized a 1200 watt lamp whereas Burlinson and coworkers used a 450 watt lamp.

and RDX in the irradiated solutions are also presented. The initial concentrations of TNT and RDX in LAP water were 35 and 22 mg/L (1.59:1.0), respectively. Each treatment group in each quadruplicate test contained five first instar daphnids, and the 2 tests were performed simultaneously.

Table 12. ACUTE TOXICITY TO D. MAGNA OF LAP WATER WITH DIFFERENT DEGREES OF PHOTOLYSIS (Test Series 1)

Flow Rate (mL/min)	Final Concentration (mg/L)		48-Hour LC50 (mg/L)[a]				
	TNT	RDX	Test 1	Test 2	Test 3	Test 4	Pooled[b]
14	0.1	0.7	19.6	20.0	24.2	22.9	21.7 (5)
25	0.2	3.4	22.6	11.7	25.4	25.7	21.8 (0)
47	0.3	7.3	25.7	27.8	24.6	24.6	23.4 (0)
100	1.8	13.7	16.0	16.1	17.9	16.7	17.6 (0)
210	11.5	20.0	12.7	17.3	14.2	12.5	13.5 (0)

[a]Based on the combined initial concentrations of 35 mg/L of TNT and 22 mg/L of RDX.
[b]Percent control mortality is shown in parentheses.

The degree of photolysis of TNT ranged from 67.1 percent in LAP water irradiated at 210 mL/min to 99.7 percent in LAP water irradiated at 14 mL/min. At these flow rates, 9.1 and 96.8 percent, respectively, of the initial RDX in solution were photolyzed. The degree of photolysis of these compounds increased with decreasing flow rates because the residence time of LAP water in the reactor increased as the flow rate decreased.

The replicate tests on each photolyzed solution produced different LC50s, and the variation was considerable in some cases; however, with the exception of Test 2, the LC50s of LAP water increased as the concentrations of TNT and RDX decreased. The pooled LC50s show that, in general, the toxicity of LAP water decreased as the irradiation flow rate decreased from 210 to 47 mL/min, but did not change markedly when the flow rate was reduced from 47 to 14 mL/min.

To confirm this region of toxicological stability, we performed a second similar series of tests--this time with LAP water that had been irradiated at flow rates of 4.5 to 99 mL/min. Table 13 presents the results. In this series of tests, we did not obtain LC50s for LAP water irradiated at 5.4 to 34 mL/min because we did not test concentrations high enough to immobilize at least 50 percent of the daphnids. The LC50s of LAP water irradiated at 99 and 53 mL/min in Test Series 2 were about the same as the LC50s of LAP water irradiated at 100 and 47 mL/min in Test Series 1. For LAP water irradiated at flow rates closely corresponding

to rates used in Test Series 1, we obtained approximately the same final concentrations of TNT and RDX. These results indicated a consistency in irradiation procedures.

Table 13. ACUTE TOXICITY TO D. MAGNA OF LAP WATER WITH DIFFERENT DEGREES OF PHOTOLYSIS (Test Series 2)

Flow Rate (mL/min)	Final Concentration (mg/L) TNT	RDX	48-Hour LC50 (mg/L)[a]	Immobilization at the Highest Concentration[b] %
5.4	0.01	0.003	>28.2 (15)	40
10.0	0.02	0.07	>28.2 (15)	20
18.4	0.09	2.3	>28.2 (20)	30
34.0	0.18	6.5	>28.2 (30)	30
53.0	0.38	9.3	27.1 (0)	65
73.0	0.45	10.5	22.7 (20)	75
99.0	1.27	12.9	15.0 (10)	95

[a]Based on the combined concentrations of 35 mg/L of TNT and 22 mg/L of RDX. Percent control mortality is shown in parentheses.
[b]28.2 mg/L.

The LC50s were not useful for identifying the region of toxicological stability, so we examined the response data obtained for the highest concentration (28.2 mg/L) of LAP water tested in Test Series 2. This concentration affected increasingly fewer organisms as the irradiation flow rate decreased from 99 to 34 mL/min, but at lower flow rates the percentage of organisms affected was about the same as at 34 mL/min. We interpreted these results to indicate that stabilization had occurred. After discussing the data from both series of tests with USAMRDC, we decided to define photolyzed LAP water as a solution containing 35 mg/L of TNT and 22 mg/L of RDX before irradiation and 0.1 to 0.2 mg/L of TNT and 2.3 to 4.6 mg/L of RDX after irradiation.

Task 2 - Acute Toxicity of LAP Water and TNT to Organisms From Three Trophic Levels

Toxicity to Fish. All of the tests with fish were performed in duplicate, except that the "duplicate" tests with the bluegill sunfish and rainbow trout on TNT were not performed simultaneously or with the same concentrations of TNT. Each species of fish was used to determine the acute toxicity of TNT and LAP water before and after these test materials were exposed to simulated sunlight.

The fish used in the tests were juveniles. The respective average weights and lengths (total) and their ranges were 1.35 g (0.96 - 1.94)

and 4.3 cm (3.8 - 4.7) for the minnows; 1.6 g (1.0 - 2.4) and 4.0 cm (3.5 - 4.7) for the bluegills; 3.6 g (1.1 - 9.1) and 6.5 cm (4.7 - 9.0) for the trout; and 2.0 g (0.7 - 4.8) and 5.6 cm (3.8 - 7.5) for the catfish.

Before it was irradiated, the TNT solution contained 90 mg/L of TNT; after irradiation, it contained 3.3 mg/L of TNT; hence, the degree of photolysis was 93.3 percent. LAP water contained 37.0 mg/L of TNT and 23.0 mg/L of RDX (TNT:RDX = 1.61:1) before it was irradiated and less than 0.05 mg/L of TNT and 4.3 mg/L of RDX after it was irradiated. Table 14 summarizes the results of the tests.

The 96-hour LC50s for TNT ranged from 1.15 mg/L (average of 1.5 and 0.8 mg/L) with rainbow trout to 2.9 with the fathead minnow and 3.0 with the bluegill sunfish (average of 3.4 and 2.6 mg/L). The 96-hour LC50s for photolyzed TNT ranged from 5.5 mg/L with the channel catfish to 18.3 mg/L with the bluegill sunfish.

LAP water was considerably more toxic to rainbow trout than to the three other species of fish. The 96-hour LC50 of LAP water ranged from 1.7 mg/L for the trout to 5.9 mg/L for fathead minnows. The channel catfish and rainbow trout were equally sensitive to photolyzed LAP water and more sensitive than the minnow and bluegill, which also showed approximately equal sensitivities to the photolyzed mixture of TNT and RDX. The results support earlier evidence that photolysis reduces the acute toxicity of LAP water and TNT.

Toxicity to Invertebrates. Table 15 presents the 48-hour LC50s and 95 percent confidence limits determined with four species of invertebrates for photolyzed and nonphotolyzed LAP water and TNT. In the tests, we used the same stock solutions of the test materials that we used in the tests with fish.

For TNT, the 48-hour LC50s ranged from 5.2 mg/L with L. variegatus to 27.0 mg/L with T. dissimilis. We were surprised to find L. variegatus so sensitive to TNT. Freshwater oligochaetes are generally considered very tolerant of organic materials because they usually inhabit highly polluted areas. The 48-hour LC50s for photolyzed TNT ranged from 6.7 mg/L with H. azteca to 19.2 mg/L with L. variegatus. Photolysis did not reduce the toxicity of TNT to H. azteca.

The 48-hour LC50s for LAP water ranged from 9.0 mg/L with L. variegatus to 31.8 mg/L with T. dissimilis. For photolyzed LAP water, the 48-hour LC50s ranged from 47.4 mg/L with H. azteca to >54.7 mg/L with T. dissimilis.

Toxicity to algae. Tables 16, 17, 18, and 19 summarize the effect of TNT, photolyzed TNT, LAP water, and photolyzed LAP water, respectively, on the population growth of the four species of algae. Population growth is expressed as the percentage difference between the mean cell concentration in the control cultures and in the treated cultures. The plus (+) sign indicates that the mean cell concentration in the cultures receiving

Table 14. ACUTE TOXICITY OF TNT AND LAP WATER BEFORE AND AFTER PHOTOLYZATION TO FOUR SPECIES OF FISH

Fish Species	Pooled 96-Hour LC50 and 95% Confidence Limits (mg/L)			
	TNT[a]	Photolyzed TNT[b]	LAP Water	Photolyzed LAP Water[b]
Fathead minnow	2.9 (2.6-3.2)	12.8 (11.6-13.9)	5.9 (5.4-6.8)	32.9 (31.5-34.4)[c]
Channel catfish	2.4 (2.0-2.7)[c]	5.5 (4.8-6.4)[c]	5.2 (4.8-5.7)[c]	17.7 (15.9-19.6)[c]
Bluegill sunfish	3.4 (3.1-3.7)[c] 2.6 (2.3-2.9)	18.3 (17.2-19.5)	4.2 (3.9-4.6)[c]	35.2 (33.6-36.7)
Rainbow trout	1.5 (1.2-1.8)[c] 0.8 (0.7-1.0)	13.9 (9.0-18.0)	1.7 (1.4-2.0)[c]	15.4 (13.5-17.4)

[a]The LC50s obtained for TNT with bluegills and rainbow trout are not pooled because the two tests with these species were performed at different times.

[b]The LC50s for photolyzed TNT are based on the concentrations of TNT in solution before irradiation, and the LC50s for photolyzed LAP water are based on the combined concentrations of TNT and RDX in solution before irradiation.

[c]Test solutions aerated.

Table 15. ACUTE TOXICITY OF TNT AND LAP WATER BEFORE AND AFTER PHOTOLYZATION TO FOUR SPECIES OF INVERTEBRATES

Invertebrate Species	Pooled 48-Hour LC50 and 95% Confidence Limits (mg/L)[a]			
	TNT	Photolyzed TNT	LAP Water	Photolyzed LAP Water
D. magna	11.7 (10.9-12.6)	16.5 (14.3-18.6)	11.6 (10.5-12.6)	52.0 (44.3-69.7)
H. azteca	6.5 (5.6- 7.5)	6.7 (5.0- 8.9)	10.4 (9.7-11.3)	47.4 (32.8-infinity)
T. dissimilis	27.0 (22.0-33.0)	25.2 (17.3-30.6)	31.8 (26.1-43.7)	>54.7 (NC)[b]
L. variegatus	5.2 (4.5- 6.0)	19.2 (16.0-22.3)	9.0 (7.8-10.1)	47.7 (41.0-54.7)

[a]For TNT and photolyzed TNT, the LC50s are based on the concentration of TNT in solution before irradiation. For LAP water and photolyzed LAP water, the LC50s are based on the combined concentration of TNT and RDX in solution before irradiation.

[b]Not calculated.

Table 16. EFFECT OF TNT ON THE CONCENTRATION OF ALGAL CELLS IN CULTURES EXPOSED FOR 14 DAYS

Concentration of TNT[a] (mg/L)	Percentage of Population Growth Stimulation (+) or Inhibition (-) Relative to Controls					
	A. flos-aquae 14 days	N. pelliculosa[c] 14 days	S. capricornutum 4 days	S. capricornutum 14 days	M. aeruginosa 4 days	M. aeruginosa 14 days
0.72		-15.9				
0.82	-3.8		-42.2	-38.8	-44.2	-26.5
3.6		+2.2				
4.1	-21.3[b]		-98.4[b]	-63.9[b]	-64.4[b]	-99.1[b]
7.2		-7.5				
8.2	-54.1[b]		-99.5[b]	-69.8[b]	-65.8[b]	-98.9
18.0		-90.1[b]				
20.5	-98.0[b]		-99.6[b]	-99.6[b]	-67.8[b]	-98.3[b]
36.0		-96.2[b]				
41.0	-98.3[b]		-99.6[b]	-99.6[b]	-53.6[b]	-99.1[b]
72.0		-97.2[b]				
82.0	-98.7[b]		-99.5[b]	-99.6[b]	-71.7[b]	-98.2[b]

[a] Nominal (based on initial stock concentration).

[b] Significantly different from controls ($p \leq 0.05$).

[c] Tested at a different time from the other species, using a different TNT stock solution.

Table 17. EFFECT OF PHOTOLYZED TNT ON THE CONCENTRATION OF ALGAL CELLS IN CULTURES EXPOSED FOR 14 DAYS

Concentration of Photolyzed TNT[a] (mg/L)	Percentage of Population Growth Stimulation (+) or Inhibition (-) Relative to Controls					
	A. flos-aquae	N. pelliculosa	S. capricornutum		M. aeruginosa	
	14 days	14 days	4 days	14 days	4 days	14 days
0.8	-2.2	-13.5	+128.3	+37.6	+16.7	+4.9
4.1	-40.1[b]	+54.5[b]	+161.0	+47.7	-17.9	-40.4[b]
8.3	-47.9[b]	+92.8[b]	+161.2	+29.6	-3.6	-75.6[b]
20.7	-56.0[b]	-95.0[b]	-68.2	+17.1	+0.03	-86.9[b]
41.4	-90.5[b]	-95.9[b]	-77.6	-65.2[b]	+8.6	-94.1[b]
82.9	-95.2[b]	-96.4[b]	-88.4	-96.9[b]	+26.6	-96.8[b]

[a]Nominal concentration of TNT before photolysis.

[b]Significantly different from controls ($p \leq 0.05$).

Table 18. EFFECT OF LAP WATER ON THE CONCENTRATION OF ALGAL CELLS IN CULTURES EXPOSED FOR 14 DAYS

Concentration of LAP Water[a] (mg/L)	Percentage of Population Growth Stimulation (+) or Inhibition (-) Relative to Controls					
	A. flos-aquae	N. pelliculosa	S. capricornutum		M. aeruginosa	
	14 days	14 days	4 days	14 days	4 days	14 days
0.6	+9.2	+122.9[b]	+309.6[b]	-3.8	-60.1[b]	-46.3[b]
2.8	-35.0	+156.9[b]	+67.2[b]	+9.4	-79.3[b]	-99.0[b]
5.5	-11.8	+146.2[b]	+18.2	+0.4	-80.9[b]	-99.9[b]
13.8	-50.6	+92.1[b]	-73.2[b]	-14.8	-97.9[b]	-100.0[b]
27.6	-77.4[b]	-88.7[b]	-85.7[b]	-99.4[b]	-37.1	-99.2[b]
55.3	-81.4[b]	-92.2[b]	-81.9[b]	-99.1[b]	+12.4	-98.6[b]

[a]Nominal combined concentrations of TNT and RDX.

[b]Significantly different from controls ($p \leq 0.05$).

Table 19. EFFECT OF PHOTOLYZED LAP WATER ON THE CONCENTRATION OF ALGAL CELLS IN CULTURES EXPOSED FOR 14 DAYS

Concentration of Photolyzed LAP Water[a] (mg/L)	Percentage of Population Growth Stimulation (+) or Inhibition (-) Relative to Controls					
	A. flos-aquae 14 days	N. pelliculosa 14 days	S. capricornutum 4 days	S. capricornutum 14 days	M. aeruginosa 4 days	M. aeruginosa 14 days
0.5	-2.9	+5.9	+6.7	+1.6	-13.2[b]	-24.6
2.5	+4.0	+7.1	+8.8	-1.0	-24.9[b]	-50.3
4.9	-5.2	+9.5	+1.2	-6.7	-32.6[b]	-32.1
12.4	-16.5	+6.6	-6.8[b]	-13.0	-42.9[b]	-45.7
24.7	-23.2	-28.7[b]	-30.8[b]	-3.8	-47.3[b]	-34.9
49.4	-36.0	+2.2	-97.0[b]	-29.8[b]	-35.1[b]	-93.9[b]

[a]Nominal combined concentrations of TNT and RDX before photolysis.

[b]Significantly different from controls ($p \leq 0.05$).

a specific treatment was larger than that in the control cultures and that population growth was stimulated. The negative (-) sign signifies that the opposite occurred and that the growth of the cultures receiving a specific treatment was inhibited. Concentrations that caused statistically significant ($p \geq 0.05$) effects are identified.

As stated in the Methods Section, we determined the concentration of the algal cells in the cultures of S. capricornutum and M. aerguinosa on the 4th and 14th (last) days of the test. However, with A. flos-aquae and N. pelliculosa we performed this measurement only on the 14th day because homogenization of the cultures (required to separate the cells) affected the growth rate of these species.

The concentrations used in each test do not conform to a regular pattern because of the dilution procedure that we used, which was the one recommended at an EPA-sponsored algal assay workshop held in Corvallis, Oregon in 1974. After preparing and analyzing the stock solutions for the test material, we used a dilution series of 100, 50, 25, 10, 5, and 1 percent full-strength stock solution. Thus, if a stock solution contained 82 mg/L of TNT, the nominal test concentrations were 82, 41, 20.5, 8.2, 4.1, and 0.82 mg/L. This dilution procedure produced fairly large gaps between the 3rd and 4th highest concentrations and between the two lowest concentrations.

The predominant effect of TNT on algal population growth was inhibition (Table 16). N. pelliculosa showed 2.2 percent stimulation at 3.8 mg/L; however, the effect was not statistically significant. The lowest concentration that caused a significant effect was 4.1 mg/L. This concentration affected only S. capricornutum and M. aeruginosa. A. flos-aquae did not show a significant response until the concentration reached 8.2 mg/L.

N. pelliculosa was not tested at the same concentrations as the other algae, so a direct comparison of its relative sensitivity cannot be made. This species showed significant inhibition (90.1 percent) at 18 mg/L, but not at the next lower concentration of 7.2 mg/L.

At the three lowest tested concentrations, S. capricornutum showed some recovery on the 14th day of effects observed on the 4th day. M. aeruginosa also showed some recovery between the 4th and 14th day at 0.82 mg/L; however, the degree of inhibition was not statistically significant on either day, so we are uncertain if recovery actually occurred.

Photolyzed TNT stimulated as well as inhibited the growth of all species except A. flos-aquae, which was inhibited only (Table 17). At the lowest concentration of 0.8 mg/L, there were no statistically significant effects. At 4.1 mg/L (the second lowest concentration), photolyzed TNT caused 54.5 percent stimulation in N. pelliculosa and 40.4 percent inhibition in M. aeruginosa. These effects were statistically significant. The lowest concentration of photolyzed TNT that caused statistically significant effects in all species was 41.4 mg/L.

LAP water both stimulated and inhibited the growth of two of the algal species, N. pelliculosa and S. capricornutum (Table 18). In A. flos-aquae and M. aeruginosa, the stimulatory effects were not statistically significant. The tested concentrations were 0.6, 2.8, 5.5, 13.8, 27.6, and 55.3 mg/L.

In N. pelliculosa, stimulation ranging from 92.1 to 156.9 percent occurred at the four lowest concentrations; at the two highest concentrations, growth was significantly inhibited. At all concentrations, the results in N. pelliculosa were statistically significant. In S. capricornutum, statistically significant stimulation of up to about 310 percent occurred at the two lowest concentrations after four days of exposure. However, population growth declined after the 4th day, and by the 14th day the concentrations were about the same as in the control flasks. S. capricornutum was significantly inhibited after four days of exposure to 13.8 to 55.3 mg/L of LAP water and after 14 days of exposure to 27.6 and 55.3 mg/L.

A. flos-aquae showed statistically significant effects (inhibition) only at the two highest test concentrations. After four days of exposure, M. aeruginosa was significantly inhibited at all concentrations up to 13.8 mg/L. Unexplainably, the degree of inhibition at 27.6 mg/L was considerably lower than at the lower concentrations, and at 55.3 mg/L the alga showed 12.4 percent growth stimulation. The effects at 27.6 and 55.3 mg/L were not statistically significant. After 14 days of exposure, M. aeruginosa showed 46.3 percent inhibition at the lowest tested concentration and at least 98 percent inhibition (significant) at all other concentrations.

Photolyzed LAP water did not cause as much stimulation or inhibition as LAP water at similar concentrations (Table 19). Stimulatory effects were not statistically significant in any of the species. The lowest concentrations that caused statistically significant inhibition after 14 days of exposure were 49.4 mg/L in A. flos-aquae and M. aeruginosa, 24.7 mg/L N. pelliculosa, and 12.4 mg/L in S. capricornutum.

During the algal assays on nonphotolyzed TNT and LAP water, we noticed that the test solutions became reddish-brown, which indicated TNT photolysis. To determine the extent of photolysis, we monitored the TNT concentrations in a test on TNT. The monitoring data are shown below:

	Concentration of TNT (mg/L)		
Flask	Day 0	Day 4	Day 14
2	0.8	Trace	Trace
5	20.8	15.6	5.3
7	83.0	78.0	59.2

These data indicate that the toxicity estimates for nonphotolyzed TNT and LAP water are unreliable. The observed effects cannot be attributed only to TNT or TNT and RDX; possible effects of their phototransformation

products must be considered. The decline in the concentration of TNT in the culture flasks could have had an impact on the effects observed on Days 4 and 14. Recovery of the algal populations on Day 14 may have been a reflection of reduced TNT concentrations rather than acclimation to the presence of TNT.

We feel that the results of the algal assays should be interpreted carefully. The bottle test is inappropriate for evaluating photosensitive substances. Unfortunately, chemostatic tests with algae have not been perfected. Such a test would have been preferable.

Task 3 - Effect of Water Quality on the Acute Toxicity of LAP Water and Photolyzed LAP Water

Table 20 shows the effect of water temperature, hardness, and pH on the 96-hour LC50 obtained with bluegills for LAP water.

Table 20. EFFECTS OF WATER TEMPERATURE, HARDNESS, AND pH ON THE ACUTE TOXICITY OF LAP WATER TO BLUEGILLS

Water Quality Parameter	Level		96-Hour LC50 and 95% Confidence Limits (mg/L)
	Desired	Actual	
Temperature[a] (°C)	15	15.8	3.2 (2.7-3.7)
	20	20.2	2.5 (2.2-2.9)[b]
	25	24.8	4.2 (3.9-4.5)[b]
Hardness[c] (mg/L)	40	32	2.5 (2.2-2.9)[b]
	100	87	3.2 (2.8-3.6)[b]
	250	224	2.9 (2.5-3.4)
pH[d]	6	5.9	2.4 (2.1-2.7)[b]
	7	7.1	2.5 (2.2-2.9)[b]
	8	8.1	2.9 (2.5-3.4)[b]

[a] Nominal pH and hardness were 7.0 ($\bar{x}$ = 7.1, range = 7.1-7.1) and 40 mg/L ($\bar{x}$ = 34.7, range = 32.0-36.0), respectively.
[b] Test solutions aerated.
[c] Nominal temperature and pH were 20°C ($\bar{x}$ = 20.4, range = 20.0-20.8) and 7.0 ($\bar{x}$ = 7.2, range = 7.1-7.3), respectively.
[d] Nominal temperature and hardness were 20°C ($\bar{x}$ = 20.0, range = 20.0-20.0) and 40 mg/L ($\bar{x}$ = 32.0, range = 32.0-32.0), respectively.

The 96-hour LC50s obtained at three temperatures were significantly different ($p \geq 0.05$) from each other; however, temperature did not affect the toxicity of LAP water in the expected manner. Whenever temperature affects the toxicity of a chemical, the chemical usually exhibits greater

toxicity as the temperature increases. With LAP water, toxicity was lowest at the highest temperature and highest at the middle temperature.

The 96-hour LC50s obtained at the three levels of hardness were not statistically different from each other. This indicates that the toxicity of LAP water is not affected by hardness ranging from 34 to 224 mg/L as $CaCO_3$.

The 96-hour LC50s increased with increasing pH, indicating that the toxicity of LAP water decreases as the pH of the test medium shifts from acidic to basic conditions; however, the differences between LC50s were not statistically significant.

Table 21 presents the 96-hour LC50s obtained for photolyzed LAP water under several conditions of water temperature, hardness, and pH. The 96-hour LC50s obtained at the three temperatures differed significantly from each other, and increased as temperature increased. At hardness levels of 34 and 255 mg/L (as $CaCO_3$), the 96-hour LC50s were 13.0 and 13.7 mg/L, respectively; this difference between these LC50s was not statistically significant. However, at 94 mg/L hardness, the 96-hour LC50 was 16.8 mg/L, significantly higher than the values obtained at the other two hardness conditions. The 96-hour LC50s of photolyzed LAP water increased significantly with increase of the pH of the test solutions.

Table 21. EFFECTS OF WATER TEMPERATURE, HARDNESS, AND pH ON THE ACUTE TOXICITY OF PHOTOLYZED LAP WATER TO THE CHANNEL CATFISH

Water Quality Parameter	Level		96-Hour LC50 and 95% Confidence Limits (mg/L)
	Desired	Actual	
Temperature[a] (°C)	5	15.2	10.9 (9.6-12.2)[b]
	20	20.0	13.0 (11.7-14.3)[b]
	25	24.6	16.5 (15.3-17.8)[b]
Hardness[c] (mg/L $CaCO_3$)	40	20	13.0 (11.7-14.3)[b]
	100	94	16.8 (15.6-18.0)[b]
	250	255	13.7 (12.5-14.9)[b]
pH[d]	6	6.1	7.8 (5.5-11.0)
	7	7.1	13.0 (11.7-14.3)[b]
	8	7.6	16.5 (15.2-17.9)[b]

[a] Nominal pH and hardness were 7.0 ($\bar{x}$ = 7.1, range = 7.0-7.1) and 40 mg/L ($\bar{x}$ = 20.0, range = 20.0-20.0), respectively.
[b] Test solutions aerated.
[c] Nominal temperature and pH were 20°C ($\bar{x}$ = 20.5, range = 20.0-21.0) and 7.0 ($\bar{x}$ = 7.2, range = 7.1-7.3), respectively.
[d] Nominal temperature and hardness were 20°C ($\bar{x}$ = 21.0, range = 20.0-21.5) and 40 mg/L ($\bar{x}$ = 18.7, range = 18.0-20.0), respectively.

The influence of the three water quality parameters on the toxicity of photolyzed and nonphotolyzed LAP water was relatively small, particularly with nonphotolyzed LAP water. None of the water quality parameters caused the toxicity of either type of LAP water to change by more than a factor of two.

However, the results obtained from this task show that photolysis not only reduces the toxicity of LAP water (a fact already shown by earlier tasks), but also changes the toxicological properties of LAP water relative to water temperature and hardness. The toxicity of photolyzed LAP water decreased with increasing temperature, but the toxicity of nonphotolyzed LAP water did not. Photolyzed LAP water exhibited less toxicity in moderately hard water than in soft and very hard water, but nonphotolyzed LAP water was not affected by water hardness. The pH of the test solutions had a much greater effect on the toxicity of photolyzed LAP water than it had on nonphotolyzed LAP water.

Task 4 - Acute Toxicity of Photolyzed and Nonphotolyzed LAP Water to Selected Early Life Stages of the Fathead Minnow

Table 22 presents the 96-hour LC50s we obtained for photolyzed and nonphotolyzed LAP water in separate tests with five early life stages of the fathead minnow. The data confirmed the earlier conclusion that photolyzation reduces the toxicity of LAP water.

Table 22. ACUTE TOXICITY OF PHOTOLYZED AND NONPHOTOLYZED LAP WATER TO SELECTED EARLY LIFE STAGES OF THE FATHEAD MINNOW

	96-Hour LC50 and 95% Confidence Limits (mg/L)	
Life Stage	Nonphotolyzed	Photolyzed[a]
Embryo	12.5 (10.9-14.5)	>50.1
2-Day-old Fry	1.1 (0.9-1.4)	18.5 (15.0-20.9)
7-Day-old Fry	0.7 (0.6-1.0)	29.7 (28.3-32.3)
30-Day-old Fry	4.2 (3.8-4.7)	18.2 (16.6-19.7)
60-Day-old Fry	5.1 (4.6-5.6)	21.8 (20.9-22.9)

[a]The LC50s for photolyzed LAP water are based on the sum of the TNT and RDX concentrations in the mixture before irradiation.

The embryo was significantly more tolerant of both types of LAP water than any of the fry stages. We believe that the greater tolerance of the embryo may be attributable to the semipermeable chorion, which encloses the embryo. It could have limited the amount of toxic material that reached the embryos.

The 2- and 7-day-old fry exhibited about equal sensitivity to nonphotolyzed LAP water (the difference between the 96-hour LC50s was not

statistically significant). As the fry became older, they became significantly less sensitive. This age effect was not apparent with photolyzed LAP water; the order of sensitivity relative to fry age was 2-day = 30-day > 60-day > 7-day.

Table 23 shows the effect of nonphotolyzed LAP water on egg hatching success and fry survival in the fathead minnow after a total of 144 hours of exposure. These data and similar data on photolyzed LAP water (Table 24) were obtained by extending the acute toxicity tests on the eggs (embryo) until all of the live eggs hatched. Fry survival was based on the number of fry alive upon hatching. In the test on nonphotolyzed LAP water, all of the live eggs hatched within 6 days (144 hours) after exposure began. In the test on photolyzed LAP water, a few of the live eggs had not hatched by the end of the 6th day of exposure; however, they hatched by the end of the 7th day (168 hours). We did not collect data to determine the effect of either type of wastewater on the time of hatching; however, we have historical data that show that the time of hatching of control eggs can vary from 5 to 7 days; thus, we do not believe that the difference in the hatching time observed in the two tests is toxicant-related.

Table 23. EFFECT OF LAP WATER ON EGG HATCHING SUCCESS AND FRY SURVIVAL IN THE FATHEAD MINNOW AFTER 144 HOURS OF EXPOSURE

Concentration of LAP Water (mg/liter)[a]	Percentage That Hatched[b]	Percentage of Fry Survival[c]
Control	90	100.0
1.6	90	5.5
4.9	100	0.0
8.1	85	0.0
11.4	50	0.0
16.2	35	0.0
20.0	0	0.0

[a]Total nominal TNT and RDX concentration.
[b]Initial number of eggs, 20.
[c]Of total fry immediately after hatching.

Analysis of variance revealed that nonphotolyzed LAP water had a significant ($p \leq 0.001$), concentration-related effect on egg hatchability. William's test indicated that the percentages of eggs that hatched in LAP water concentrations of 11.4 to 20.0 mg/L were significantly less than the percentage of eggs that hatched in the control group.

The tested nonphotolyzed LAP water concentrations killed almost all of the fry immediately after they hatched. Only 5.5 percent of the fry

survived at the lowest tested concentrations of 1.68 mg/L. There were no survivors at the other concentrations. As expected, the Chi-Square test indicated that the effect at 1.6 mg/L was statistically significant ($p \leq 0.001$).

Table 24. EFFECT OF PHOTOLYZED LAP WATER ON EGG HATCHING SUCCESS AND FRY SURVIVAL IN THE FATHEAD MINNOW AFTER 168 HOURS OF EXPOSURE

Concentration of LAP Water[a] (mg/liter)	Percentage That Hatched[b]	Percentage of Fry Survival[c]
0	100	100.0
10	90	94.4
16	100	95.0
22.5	90	77.8
27.6	80	0.0
37.6	100	0.0
43.6	90	0.0
50.1	95	0.0

[a] Total nominal TNT and RDX concentration.
[b] Initial number of eggs, 20.
[c] Of total fry immediately after hatching.

Analysis of variance indicated that none of the concentrations of photolyzed LAP water had a significant effect on egg hatching success. The effect on fry survival was significant ($p \leq 0.004$) at concentrations of 27.6 to 50.1 mg/L according to William's test.

Task 5 - Exploratory Bioconcentration Studies

Table 25 presents the results of 4-day bioconcentration tests with four aquatic species on TNT, RDX, and a 1.6:1 mixture of TNT and RDX (LAP water). We calculated the 4-day bioconcentration factors by dividing the average amount of radioactivity found in the biological samples by the average amount of radioactivity found in the exposure medium (water).

All of the test organisms concentrated TNT more extensively than RDX. With the intact organisms, the 4-day BCFs ranged from about 200 to 450 for TNT and from 1.6 to 123 for RDX. For both compounds, the highest BCF was obtained with the alga, S. capricornutum.

With the bluegill, the BCFs for TNT were 338 and 9.5 based on analysis of the viscera and muscle tissue, respectively. The reason for the difference between the levels of radioactivity in these two tissues is unclear. The visceral sample excluded the gills and kidneys; the largest

Table 25. CONCENTRATION OF TNT AND RDX IN SELECTED AQUATIC ORGANISMS AFTER 96 HOURS OF EXPOSURE

Organism	Compound	Average Amount of Radioactivity Recovered[a]		
		Water[b]	Tissue	4-Day BCF[c]
S. capricornutum	TNT	356	161,162	453.0
D. magna	TNT	519	108,643	209.0
L. variegatus	TNT	450	91,092	202.0
Bluegill				
Viscera	TNT	453	153,017	338.0
Muscle	TNT	453	4,301	9.5
S. capricornutum	RDX	528	65,000	123.0
D. magna	RDX	676	1,096	1.6
L. variegatus	RDX	680	2,033	3.0
Bluegill				
Viscera	RDX	652	2,031	3.1
Muscle	RDX	652	1,259	1.9
Bluegill				
Viscera	TNT/RDX[d]	1,062	202,553	191.0
Muscle	TNT/RDX[d]	1,062	4,149	3.9

[a] Disintegrations per minute per gram.

[b] At beginning of test.

[c] Bioconcentration factor: tissue level/water level.

[d] 1.6:1 ratio.

"organ" in the sample was the gastrointestinal tract. No food was provided to the fish during the experiment; thus, the higher BCF could not have been caused by intake of TNT-contaminated food. It is unlikely that the higher visceral BCF was caused by ingestion of the exposure medium because freshwater fish normally do not ingest water. We believe that TNT is metabolized primarily in the liver, and that most of the radioactivity in the viscera was from the liver.

For RDX, the 4-day BCFs based on the analysis of the viscera and muscle tissue of the bluegill were 3.1 and 1.9, respectively. The BCFs are lower than the 3-day BCFs of 3.5 and 9.5 and the 28-day (or steady-state) BCFs of 4.7 and 3.7 obtained for RDX with the same bluegill tissues by Bentley and coworkers (1978).

The BCFs for the TNT/RDX mixture in bluegill viscera and muscle tissue were less than the BCFs for TNT alone even though the total concentration of radioactivity in the TNT/RDX solution was greater than that in the solutions containing TNT or RDX. At first, we thought that the difference indicated antagonism between the two compounds. However, we discovered just the opposite. By proportion, we estimated that the 1062 dpm/mL in the 1.6:1 TNT/RDX solution contained 435 dpm of carbon-14 from the labeled TNT. Using the results obtained from the experiments on the individual compounds, we calculated that exposure to 435 dpm/mL of TNT should have caused the viscera to accumulate 153,114 dpm/g of tissue and exposure to 627 dpm of RDX should have resulted in a visceral concentration of 1944 dpm/g for a total of 155,058 dpm/g. This value divided by the observed concentration of radioactivity in the TNT/RDX solution gives a BCF of 146, which is lower than the observed BCF of 191; thus, it appears that when in solution together, TNT and/or RDX tend to bioconcentrate more extensively than when they are present alone.

Task 5 was performed primarily to obtain information that would enable us to decide whether TNT should be subjected to a full-scale bioconcentration test with fish. RDX was not included in this decision process because it had already been evaluated in a full-scale test by Bentley and coworkers (1978). The currently accepted procedure for conducting a full-scale test entails exposing a group of fish to the test material for 28 days under flow-through conditions, then maintaining the remaining fish in clean, flowing water for about 14 days. Water and fish are sampled periodically during the entire test to determine the rate of uptake and clearance of the test material, and interval BCFs are calculated. The BCF of major interest and the one that is used to assess the hazard of the material through bioconcentration of the steady-state BCF, which is calculated when the concentration of the material in the fish ceases to increase.

The 4-day BCFs obtained in Task 5 were not very useful for estimating the bioconcentration potential of TNT because they represented only one point on the uptake curve for each organism, and thus did not permit extrapolation. Also, BCFs based on radioactivity recovery data alone may

overestimate the actual BCF of a compound because such data do not distinguish between the radioactivity contributions from the compound and from its transformation products.

The octanol-water partition coefficient (P) has been used frequently as an index of the propensity of organic compounds to bioconcentrate. Compounds with a Log P value of less than 3.0 are usually considered to exhibit little or no bioconcentration hazard. Using the method of Hansch (1973) and Leo (1976), we calculated for TNT a Log P value of 2.03, which suggests that it should not bioconcentrate extensively in fish.

A number of investigators (Neely et al., 1974; Chiou et al., 1977; Kenaga and Goring 1978; Veith et al., 1978) have found a direct relationship between Log P and the steady-state BCF of organic compounds in fish and have developed regression equations to express the relationship. The equation developed by Veith and coworkers (1978) is recommended by EPA (1979) for estimating the steady-state BCF for a compound in aquatic organisms that contain about 8 percent lipids. Using the Log P value determined for the compound, the equation is:

$$\text{Log BCF} = 0.76 \text{ Log P} - 0.23.$$

Using this equation, we calculated a steady-state BCF for TNT of 20.5. According to hazard evaluation schemes and guidelines developed independently by the American Society for Testing and Materials and the American Institute of Biological Sciences (Cairns and Dickson, 1978), compounds with steady-state BCFs of 10 or less are not considered to pose a bioconcentration hazard and compounds with steady-state BCFs of 100 or less have a low probability of creating such a hazard.

The calculated steady-state BCF for TNT falls in the latter range. Although calculated BCFs are associated with some uncertainties, we believe that an experimentally derived steady-state BCF for TNT will not differ significantly from the calculated one. Also, considering that TNT is photochemically unstable, we feel certain that it will not persist in the aquatic environment long enough to bioconcentrate significantly.

Phase III - Definitive Acute Toxicity Studies

Although Phase III originally comprised five tasks, we performed only Task 1 (see Experimental Approach for a description of each task). Task 2 was not performed because results obtained in Phase II indicated that the effect of water temperature, hardness, and pH on the acute toxicity of LAP water and TNT was minimal and required no further definition. Task 3 entailed performing the early life stage test with the channel catfish. This test is not an acute test; hence, the results are presented in Volume III. Task 4 was deleted because we decided that we had already adequately defined the effect of UV irradiation on the acute toxicity of LAP wastewater and TNT. Task 5 was not performed because the steady-state BCF for TNT (estimated from its Log P value) and the steady-state BCF determined

experimentally for RDX by Bentley and coworkers (1978) indicated that neither compound presented a bioconcentration hazard to aquatic organisms.

Task 1 - Determination of the Incipient LC50s of LAP Water and TNT

The incipient LC50 of a substance is defined as the concentration above which 50 percent of the test organisms cannot survive indefinitely (Sprague, 1969). The accepted procedure for determining the incipient LC50 of a substance is to conduct a time-independent acute toxicity test under flow-through conditions. Such a test is usually terminated when there is no additional mortality in any treatment group for 48 hours after the first 96 hours. Thus, the exposure period can vary with the substance and species of organism being tested.

The average concentrations of TNT in the test solutions used in each test are presented in Table 26. Table 27 summarizes the water quality monitoring data associated with the TNT tests. The mean lengths (total) and weights (plus standard deviations) of 10 control fish from each TNT test with fish are shown below:

Species	Average Length (cm)	Average Weight (g)
Fathead minnows	3.80 ± 1.38	0.90 ± 0.22
Bluegill sunfish	4.89 ± 0.78	1.47 ± 0.53
Channel catfish	5.30 ± 0.48	1.21 ± 0.22
Rainbow trout	5.00 ± 0.55	1.48 ± 0.47

Tables 28-30 present the average concentrations of TNT and RDX in the test solutions of LAP water. Table 31 presents the water quality monitoring data associated with each LAP water test. The means and standard deviations for the lengths (total) and weights of 10 controls from each LAP water test with fish are presented below:

Species	Average Length (cm)	Average Weight (g)
Fathead minnows	5.69 ± 0.37	1.38 ± 0.30
Bluegill sunfish	4.90 ± 0.48	1.52 ± 0.29
Channel catfish	5.27 ± 0.68	1.34 ± 0.26
Rainbow trout	7.20 ± 0.86	4.79 ± 1.04

Table 32 presents the pooled incipient LC50s as well as the pooled 24-, 48-, and 96-hour LC50s we obtained for TNT with four species of fish and two species of invertebrates. The LC50s are based on measured concentrations of TNT.

The incipient LC50 of TNT ranged from 1.4 to 1.9 mg/L with the fish species. This range is much narrower and lower than the range for the 96-hour LC50s (2.0 to 3.7 mg/L), which is the statistic most commonly used to express acute toxicity in fish. Of the six species of animals tested, D. magna exhibited the greatest sensitivity to TNT. With this invertebrate, we obtained an incipient LC50 for TNT of 0.19 mg/L.

Table 26. MEAN MEASURED CONCENTRATIONS[a] OF TNT USED TO DETERMINE THE INCIPIENT LC50

	Minnow			Bluegill			Catfish			Trout			D. magna			L. variegatus		
Treatment[b]	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
1	0.5	0.14	12	0.5	0.05	4	0.5	0.13	14	0.5	0.02	4	0.006	0.004	3	6.7	1.33	11
2	0.8	0.20	12	0.7	0.09	8	0.8	0.36	15	1.3	0.10	4	0.18	0.30	3	9.8	1.29	11
3	2.5	0.29	14	2.6	0.30	6	3.0	0.83	13	3.7	0.57	2	0.21	0.02	4	15.2	0.93	11
4	4.8	0.55	9	5.0	1.10	2	4.4	1.47	10				2.6	0.19	4	19.8	1.40	11
5	6.0	0.83	7	6.0	0.35	2	6.2	0.97	7				4.4	0.33	3	28.9	2.79	10
6	9.1	0.18	4	8.8	0.78	2	8.0	1.25	7									

[a]Expressed in mg/L.

[b]Control not listed; it was not analyzed for TNT.

SD = Standard deviation
N = Number of samples analyzed

Table 27. WATER QUALITY CHARACTERISTICS OF THE TEST SOLUTIONS USED IN THE TESTS TO DETERMINE THE INCIPIENT LC50 OF TNT

Water Quality Parameter	Minnow			Bluegill			Catfish			Trout			D. magna			L. variegatus		
	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range
Temperature (°C)	4	22	20-25	2	20.8	20.5-21.0	2	20.2	20.0	4	13.0	10-14	3	20.0	20.0	2	20.0	20.0
Hardness (mg/L as $CaCO_3$)	1	20	—	1	20	—	1	15	—	1	55	—	1	42	—	1	20	—
Alkalinity (mg/L as $CaCO_3$)	1	20	—	1	20	—	1	25	—	1	55	—	1	50	—	1	17	—
Dissolved Oxygen (mg/L)	21	7.2	6.6-8.0	18	7.8	7.2-8.6	10	7.6	7.1-8.2	7	9.3	8.9-9.8	14	8.8	8.4-9.8	17	8.6	8.4-8.8
Conductivity (μmhos/cm)	1	34	—	1	40	—	1	50	—	1	137	—	1	128	—	1	44	—
pH	11	6.9	6.5-7.4	10	7.4	7.0-8.3	4	7.4	6.8-8.0	7	7.8	7.5-8.4	10	7.6	7.4-7.7	11	7.6	7.2-8.3

Table 28. MEAN CONCENTRATIONS (MG/L) OF TNT AND RDX IN THE TEST SOLUTIONS USED TO DETERMINE THE INCIPIENT LC50 OF LAP WATER FOR FATHEAD MINNOWS AND BLUEGILLS

	Minnow					Bluegill				
Treatment[a]	N[b]	TNT	RDX	Total[c]	Ratio	N	TNT	RDX	Total	Ratio
1	14	0.43 (0.07)[d]	0.32 (0.05)	0.76	1.34:1	8	0.51 (0.04)	0.40 (0.28)	0.91	1.28:1
2	14	1.52 (0.19)	1.15 (0.18)	2.68	1.32:1	10	1.86 (0.11)	1.44 (0.10)	3.29	1.29:1
3	15	2.68 (0.35)	1.95 (0.30)	4.63	1.37:1	5	3.12 (0.13)	2.20 (0.23)	5.32	1.42:1
4	14	3.52 (0.71)	2.58 (0.56)	6.10	1.36:1	3	4.30 (0.56)	2.87 (0.15)	7.17	1.50:1
5	4	6.03 (0.73)	4.45 (0.23)	10.48	1.36:1	2	6.45 (0.21)	4.20 (0.14)	10.65	1.54:1

[a]Control not listed, it was not analyzed for TNT or RDX.

[b]Number of samples analyzed.

[c]Values used to estimate all LC50s.

[d]Values in parentheses represent the standard deviations of the population response.

Table 29. MEAN CONCENTRATIONS (MG/L) OF TNT AND RDX IN THE TEST SOLUTIONS USED TO DETERMINE THE INCIPIENT LC50 OF LAP WATER FOR CHANNEL CATFISH AND RAINBOW TROUT

	Channel Catfish					Rainbow Trout				
Treatment[a]	N[b]	TNT	RDX	Total[c]	Ratio	N	TNT	RDX	Total	Ratio
1	14	0.41 (0.12)[d]	0.31 (0.04)	0.72	1.32:1	7	0.15 (0.10)	0.17 (0.02)	0.32	0.88:1
2	18	1.46 (0.19)	1.05 (0.19)	2.51	1.39:1	13	0.51 (0.10)	0.39 (0.07)	0.91	1.31:1
3	13	2.70 (0.16)	1.83 (0.11)	4.52	1.48:1	5	1.67 (0.38)	1.34 (0.21)	3.01	1.25:1
4	7	4.08 (0.44)	2.68 (0.20)	6.76	1.52:1	4	2.94 (0.43)	2.18 (0.21)	5.12	1.35:1
5	4	6.13 (0.12)	3.93 (0.11)	10.06	1.56:1	4	3.35 (0.30)	2.26 (0.18)	5.61	1.48:1

[a]Control not listed, it was not analyzed for TNT or RDX.

[b]Number of samples analyzed.

[c]Values used to estimate all LC50s.

[d]Values in parentheses represent the standard deviations of the population response.

Table 30. MEAN CONCENTRATIONS (MG/L) OF TNT AND RDX IN THE TEST SOLUTIONS USED TO DETERMINE THE INCIPIENT LC50 OF LAP WATER FOR D. MAGNA AND L. VARIEGATUS

	Daphnia magna					Lumbriculus variegatus				
Treatment[a]	N[b]	TNT	RDX	Total[c]	Ratio	N	TNT	RDX	Total	Ratio
1	7	—[d]	—	0.1[f]	—	14	4.51 (0.32)	3.0 (0.23)	7.51	1.50:1
2	7	0.26 (0.12)[e]	0.24 (0.02)	0.51	1.08:1	14	9.28 (0.79)	6.01 (0.52)	15.29	1.54:1
3	9	0.66 (0.24)	0.52 (0.14)	1.18	1.27:1	14	13.3 (1.56)	8.72 (1.01)	22.06	1.52:1
4	6	2.51 (1.19)	1.65 (0.78)	4.16	1.52:1	14	18.77 (1.47)	12.43 (0.88)	31.19	1.51:1
5	8	4.01 (1.23)	2.70 (0.74)	6.78	1.48:1					

[a]Control not listed, it was not analyzed for TNT or RDX.

[b]Number of samples analyzed.

[c]Values used to estimate all LC50s.

[d]Concentrations of TNT and RDX too low to quantitate accurately.

[e]Values in parentheses represent the standard deviations of the population response.

[f]Estimated.

Table 31. WATER QUALITY CHARACTERISTICS OF THE TEST SOLUTIONS USED IN THE TESTS TO DETERMINE THE INCIPIENT LC50 OF TNT LAP WATER

Water Quality Parameter	Minnow			Bluegill			Catfish			Trout			D. magna			L. variegatus		
	N[a]	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range
Temperature (°C)	3	20.3	20–21	2	20.5	20–21.5	2	20.5	19–21	4	12.9	11.5–15.0	2	19.5	19–20	2	19.5	19–20
Hardness (mg/L as $CaCO_3$)	1	18	—	1	17	—	1	20	—	1	101	—	1	42	—	1	42	—
Alkalinity (mg/L as $CaCO_3$)	1	20	—	1	20	—	1	25	—	1	110	—	1	50	—	1	50	—
Dissolved Oxygen (mg/L)	15	8.0	7.1–8.9	9	7.9	7.5–8.2	9	7.7	6.6–8.2	12	9.1	5.2–11.6	10	8.7	8.5–8.8	8	8.4	8.2–8.7
Conductivity (μmhos/cm)	1	46	—	1	44	—	1	47	—	1	308	—	1	128	—	1	128	—
pH	10	7.5	7.0–8.1	9	7.2	7.1–7.3	9	7.5	7.4–7.6	10	7.9	7.6–8.2	12	7.6	7.4–7.9	8	7.45	7.4–7.5

[a]Number of analyses performed.

Table 33 presents for LAP water the pooled incipient LC50s and the pooled 24-, 48-, and 96-hour LC50s obtained with the same species of animals used to evaluate TNT. The LC50s are based on the sum of the mean measured concentrations of TNT and RDX in the test solutions.

Table 32. ACUTE TOXICITY OF TNT TO SELECTED SPECIES OF FISH AND INVERTEBRATES EXPOSED FOR APPROXIMATELY 14 DAYS UNDER FLOW-THROUGH CONDITIONS

	Pooled LC50 (mg/L)				95% Confidence Limits for
Test Species	24 hr	48 hr	96 hr	Incipient[a]	Incipient LC50
Fathead minnow	5.9	5.9	3.7	1.5 (384)	0.9 - 2.5
Bluegill sunfish	3.4	2.6	2.5	1.4 (312)	0.8 - 2.5
Channel catfish	7.4	5.6	3.3	1.6 (288)	0.9 - 3.0
Rainbow trout	2.1	2.0	2.0	1.9 (240)	1.3 - 3.3
D. magna	>4.4	>4.4	1.2	0.19 (192)[b]	0.12- 1.0
L. variegatus	>29.0	>29.0	>29.0	13.9 (336)	12.6 -15.1

[a]Total hours of exposure shown in parentheses.
[b]Control mortality - 3.3%.

Table 33. ACUTE TOXICITY OF LAP WATER TO SELECTED SPECIES OF FISH AND INVERTEBRATES EXPOSED FOR APPROXIMATELY 14 DAYS UNDER FLOW-THROUGH CONDITIONS

	Pooled LC50 (mg/L)				95% Confidence Limits for
Test Species	24 hr	48 hr	96 hr	Incipient[a]	Incipient LC50
Fathead minnow	5.7	5.4	4.8	1.6 (336)	0.8 - 4.5
Bluegill sunfish	4.7	3.8	3.4	3.3 (264)	3.1 - 3.4
Channel catfish	6.2	5.3	4.5	2.3 (360)	1.9 - 2.5
Rainbow trout	2.2	1.7	1.5	1.5 (264)	0.9 - 3.0
D. magna	>6.8	>6.8	0.36	0.17 (192)[b]	0.1 - 1.0
L. variegatus	>31.2	>31.2	27.4	16.2 (336)	14.5 -17.8

[a]Total hours of exposure shown in parentheses.
[b]Control mortality - 16.7%.

The incipient LC50s of LAP water ranged from 0.17 mg/L with D. magna to 16.2 mg/L with L. variegatus. In the fish species, they ranged from 1.5 mg/L with trout to 3.3 mg/L with bluegill. The incipient LC50 obtained with trout was significantly ($\leq$ 0.05) lower than that obtained with the bluegill, but not significantly different from those obtained with the minnow and catfish.

The incipient LC50 obtained with D. magna for LAP water is questionable. Although the samples of test solutions from the tanks representing the lowest treatment were analyzed for TNT and RDX, the concentration of these compounds were too low to achieve accurate quantitation. The average total concentration of TNT and RDX in the second lowest treatment was. 0.51 mg/liter; thus, the incipient LC50 is not within the known chemically measured range of concentrations. However, we believe that the observed incipient LC50 is close to the one we would have obtained if we had been able to accurately measure TNT and RDX in the lowest treatment level. Our reasoning follows.

A logarithmic plot of the nominal against the measured concentrations showed a good correlation between these concentrations (e.g., all points were on or almost on a straight line). From this line we estimated that the lowest treatment level would have contained about 0.1 mg/L of TNT and RDX if these compounds could have been measured. At the lowest treatment level, mortality was 40 percent at the end of the test; at the next highest concentration (0.51 mg/L), it was 96 percent. The difference between 96 and 50 percent mortality and between 0.5 and 0.17 mg/L is 82 percent of the difference between 96 and 40 percent mortality and between 0.5 and 0.1 mg/L; thus, 0.17 mg/L is not an unreasonable estimate of the incipient LC50 for D. magna.

Tables 28-30 show that the observed ratio of TNT to RDX in the test solutions was less than 1.6:1. Listed below for each test (identified by test species) are the number of fresh stock solutions prepared and the mean and standard deviation of the value obtained by dividing the measured concentration of TNT by the measured concentration of RDX in each stock solution.

Test Species	Number of Stock Solutions	Mean Dividend ± Standard Deviation
Fathead minnow	4	1.56 ± 0.048
Bluegill sunfish	3	1.62 ± 0.110
Channel catfish	6	1.58 ± 0.056
Rainbow trout	4	1.55 ± 1.032
D. magna	2	1.54 ± 0.071
L. variegatus	3	1.54 ± 0.02

These data indicate that the ratio of TNT to RDX in the stock solutions averaged somewhat below 1.6:1 in all tests except the tests performed with bluegills. The average difference between the mean observed and the desired ratio was 2.6 percent of the desired ratio, we consider this difference to be acceptable.

The ratios of TNT to RDX in the test solutions were lower than the desired ratio by more than 10 percent in many instances. This effect appeared to be most pronounced in the lower test concentrations (see Tables 28-30). Analysis of the toxicant monitoring data from the test

with fathead minnows showed that in the test solutions, the concentrations of TNT were always lower than expected and the concentrations of RDX were always higher than expected (see below).

Average Observed Concentration of TNT and RDX (mg/L)	TNT Concentration (mg/L)		RDX Concentration (mg/L)	
	Calculated	Observed	Calculated	Observed
0.76	0.46	0.43	0.29	0.32
2.68	1.63	1.52	1.04	1.15
4.63	2.82	2.68	1.81	1.95
6.10	3.75	3.52	2.38	2.58
10.48	6.39	6.03	4.09	4.45

We calculated the expected concentrations in the test solutions by proportion, using the average measured concentrations of TNT and RDX in the stock solutions. The lower-than-expected amounts of TNT in the test solutions may have been caused by photolysis; however, the reason for the higher than expected concentrations of RDX is not known.

The 1.6:1 ratio established for LAP water was based on the analysis of LAP wastewater samples obtained directly from the discharge pipes at the ammunition plant. Because of the instability of TNT in the aquatic environment, we believe that most aquatic organisms would be exposed to a lower ratio of TNT to RDX, and that although it would have been desirable to have tested a 1.6:1 mixture, the ratios of the mixtures actually tested were not so different as to render the toxicological data invalid.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

From the data presented in this report, we conclude that:

- TNT and synthetic LAP wastewater (LAP water) are toxic to some aquatic organisms at concentrations less than 1.0 mg/L.
- Exposure of TNT, LAP wastewater, and LAP water to simulated sunlight (and presumably natural sunlight) reduces their toxicity to aquatic organisms. The reduction in toxicity of LAP water appears to be caused by a decrease in the concentration of TNT.
- The toxicity of photolyzed LAP water and TNT is affected by the pH at which these substances are photoirradiated; however, the effect is not appreciable.
- The acute toxicity of LAP wastewater and LAP water appears to be caused primarily by TNT.
- 1,3,5-trinitrobenzene, 2,4,6-trinitrobenzaldehyde, 2,4,6-trinitrobenzonitrile, and 4,6-dinitroanthranil exhibited greater acute toxicity than TNT did. Depending on the initial concentration of TNT, it is unlikely that any of these phototransformation products of TNT are produced in sufficient quantities to cause acute effects.
- The acute toxicity of TNT is slightly antagonized by RDX.
- Water temperature, hardness, and pH can affect the toxicity of LAP water, TNT, and, presumably, LAP wastewater; however, the magnitude of the effect is relatively small and probably environmentally insignificant.
- TNT and RDX are sorbed and concentrated in aquatic organisms; however, the degree of bioconcentration is low. Neither compound shows a high propensity to bioconcentrate.

Recommendations

This portion of the investigation of the toxicity of TNT wastewaters to aquatic organisms has shown that synthetic LAP wastewater can cause acute effects in aquatic organisms at concentrations as low as about 0.2 mg/L, and that the toxicity of the wastewater is probably caused by TNT. The data presented in this report are not sufficient to estimate the safe concentration of TNT. Chronic toxicity data are needed to estimate the safe concentration. To obtain the necessary chronic toxicity data, we recommend implementing all tasks in Phase IV of the general experimental approach.

LITERATURE CITED

Bentley, R.E., J.W. Dean, S.J. Eils, T.A. Hollister, G.A. LeBlanc, S. Sauter, and B.H. Sleight. 1978. Laboratory Investigation of the Toxicity of Cyclotrimethylene Trinitramine (RDX) to Aquatic Organisms. Final Report, USAMRDC Contract No. DAMD17-74-C-4101. E.G.&G. Bionomics, Wareham, Massachussetts. 83 pp.

Burlinson, N.E., L.A. Kaplan, and C.E. Adams. 1973. Photochemistry of TNT: Investigation of the "Pink Water" Problem. Report No. AD 769 760. Naval Ordinance Laboratory, White Oak, Maryland. 14 pp.

Cairns, J. Jr., and K.L. Dickson. 1978. Estimating the Hazard of Chemical Substances to Aquatic Life. American Society of Testing and Materials. STP 657. 278 pp.

Chiou, C.T., V.H. Freed, D.W. Schmedding, and R.L. Kohnert. 1977. Partition coefficient and bioaccumulation of selected organic chemicals. Environ. Sci. Technol. 11:475-478.

Dilley, J.V., C.A. Tyson, and G.W. Newell. 1979. Mammalian Toxicological Evaluation of TNT wastewater. Volume II. Acute and Subacute Mammalian Toxicity of TNT and the LAP Mixture. Final Report. SRI International, Contract DAMD17-76-C-6050. 391 pp.

Draper, N.R., and H. Smith. 1968. Applied Regression Analysis. John Wiley and Sons, New York.

Environmental Protection Agency. 1971. Algal Assay Procedures: Bottle Test. National Eutrophication Research Program, Corvallis, Oregon. 82 pp.

Environmental Protection Agency. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Ecological Research Service, 660/3-75-009. 61 pp.

Environmental Protection Agency. Water Quality Criteria, Federal Register. Vol. 44, No. 52, 15 March 1979.

Gilman, H. (ed.). 1941. Organic Synthesis. Collective Vol. 1. 1,3,5-Trinitrobenzene. John Wiley and Sons, Inc., New York, pp. 541-542.

Gring, D.M. 1971. Biological Effects of Trinitrotoluene (TNT). Ph.D. Thesis, Department of Zoology, Indiana University.

Hansch, C., A. Leo, S.H. Ungar, K.H. Kim, D. Nikaitani, and E.J. Lien. 1973. Aromatic substituent constants for structure-activity correlations. J. Med. Chem. 16: 1207-1216.

Huff, B.L., W.D. Duckert, P.G. Barding, J.H. Wheeler, T.M. Hogan, and R.B. Bogardus. 1975. Aquatic Field Surveys at Radford, Holsten, Volunteer, and Milan Army Ammunition Plants. Volume IV - Milan. Final Report, USAMRDC Contract No. DAMD17-74-C-4138. Wapora, Inc., Washington, D.C. 107 pp.

Kenega, E.E., and C.A.I. Goring. 1978. Relationship between water solubility, soil-sorption, octanol-water partitioning, and bioconcentration of chemicals in biota. Presented at the Third ASTM Symposium on Aquatic Toxicology, New Orleans, 17-18 October.

LeClerc, E. 1960. The self-purification of streams and the relationship between chemical and biological tests. In. Proc, Second Symposium on Treatment of Wastewaters, Pergamon Press, London pp. 231-317.

Leo, A. 1976. Symposium on Nonbiological Transport and Transformation of Pollutants on Land and Water. National Bureau of Standards, May 1976.

Marking, L.L., and V.K. Dawson. 1975. Method for Assessment of Toxicity or Efficacy of Mixtures of Chemicals. Investigations in Fish Control Series, Report No. 67. U.S. Dept. of the Interior, Fish and Wildlife Service.

Matthews, E.R., E.H. Rex, R.L. Taylor, and J.F. Newell. 1954. Laboratory Studies Pertaining to the Treatment of TNT Wastes. AEC Report No. TID-6268. 18 pp.

Mount, D.I. 1968. Chronic toxicity of copper to the fathead minnow (Pimephales promelas). Water Res. 2:215-223.

Mount, D.I., and W.A. Brungs. 1967. A simplified dosing apparatus for fish toxicological studies. Water Res. 1:21-29.

Nay, M.W., Jr., C.W. Randall, and P.H. King. 1972. Factors affecting color development during treatment of TNT waste. Indust. Wastes (Sept./Oct.), pp. 20-29.

Neely, W.B., D.R. Branson, and G.E. Blau. 1974. Partition coefficient to measure bioconcentration potential of organic chemicals in fish. Environ. Sci. Technol. 8:1113-1115.

Pearson, J.G., L.P. Glennon, J.J. Barkley, and J.W. Highfill. 1979. An Approach to the Toxicological Evaluation of a Complex Industrial Wastewater: In Aquatic Toxicology, ASTM STP 667. L.L. Marking and R.A. Kimmerle, Eds., pp. 284-301.

Pedersen, G. 1970. Evaluation of Toxicity of Selected TNT Wastes on Fish. Phase I - Acute Toxicity of alpha TNT to Bluegills. Sanitary Engineering Special Study No. 24-007-70/71. U.S. Army Environmental Hygiene Agency, Edgewood Arsenal, MD. 35 pp.

Ruchhoft, C.C., M. LeBosquet, Jr., and W.G. Meckler. 1945. TNT Wastes from Shell-loading Plants. Ind. Eng. Chem. 37:937-943.

Schott, C.D., and E.G. Worthley. 1974. The Toxicity of TNT and Related Wastes to an Aquatic Flowering Plant, Lemna perpusilla Torr. Defense Technical Information Center, Report No. AD-778158 Edgewood Arsenal, Aberdeen Proving Ground, Maryland.

Shoaf, W.T., and B.W. Lium. 1976. Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide. Limnol. Oceanog. 21:926-928.

Sitzman, M.E., and J.C. Dacons. 1973. Formation of 2,4,6-trinitrobenzonitrile and 4-chloro-5, 7-dinitro-2-(2,4,6-trinitrophenyl)-quinazoline-1-oxide by the action of nitrosyl chloride on 2,3,4-trinitrotoluene. J. Org. Chem. 38:5363-4367.

Smock, L.A., D.L. Stoneburner, and J.R. Clark. 1976. The Toxic Effects of Trinitrotoluene (TNT) and its Primary Degradation Products on Two Species of Algae and the Fathead Minnow. Water Res. 10537-543.

Spanggord, R.J., B.W. Gibson, R.G. Keck, and G.W. Newell. 1978. Mammalian Toxicological Evaluation of TNT Wastewater (Pink Water). Volume I - Chemistry Studies. Draft Final Report, USAMRDC Contract No. DAMD17-76-C-6050. SRI International, Menlo Park, California. 315 pp.

Splitter, J.S., and M. Calvin. 1955. The photochemical behavior of some s-nitrostilbenes. J. Org. Chem. 20:1086-1115.

Sprague, J.B. 1969. Measurement of Pollutant Toxicity to Fish. I. Bioassay Methods for Acute Toxicity. Water Res. 3:793-821.

Stillwell, J.M., D.C. Cooper, M.A. Eischen, M.C. Matthews, B.E. Sherwood, and T.B. Stanford. 1976. Aquatic Field Studies at Joliet Army Ammunition Plant. Final Report, Volume I, Phase II, USAMRDC Contract No. DAMD17-74-C-4123. Battelle-Columbus Laboratories, Columbus, Ohio. 130 pp.

Sullivan, J.H., Jr., H.D. Putnam, M.A. Keirn, J.C. Nichols and J.T. McClave. 1979. A Summary and Evaluation of Aquatic Environmental Data in Relation to Establishing Water Quality Criteria for Munitions Unique Compounds. Part 4: RDX and HMX. Final Report Contract No. DAMD17-77-C-7207, U.S. Army Medical Research and Development Command, Ft. Detrick, Maryland.

Veith, G.D., et al. 1978. An Evaluation of using Partition Coefficients and Water Solubility to Estimate Bioconcentration Factors for Organic Chemicals in Fish. Manuscript. USEPA, Duluth Laboratory, Duluth, Minnesota.

Weitzel, R.L., P.B. Simon, D.E. Jerger, and J.E. Shenk. 1975. Aquatic Field Survey at Iowa Army Ammunition Plant. Final Report, USAMRDC Contract No. DAMD17-74-C-4124. Environmental Control Technology Corporation, Ann Arbor, Michigan. 145 pp.

Won, W.D., Louis H. DiSalvo, and J. Ng. 1976. Toxicity and Mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. Appl. Environ. Microbiol. 31:576-580.

DISTRIBUTION LIST

25 copies	Commander US Army Medical Bioengineering Research and Development Laboratory ATTN: SGRD-UBG Fort Detrick Frederick, MD 21701
4 copies	USAMRDC (SGRD-RMS) Fort Detrick Frederick, MD 21701
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDA Cameron Station Alexandria, VA 22314
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20014
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234
1 copy	Commander US Army Medical Bioengineering Research and Development Laboratory ATTN: SGRD-UBD-A/Librarian Fort Detrick Frederick, MD 21701